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13. ABSTRACT (Maximum 200 Words) The EGFR receptor tyrosine kinase is dysfunctional in a wide range of solid human tumors including prostate carcinomas. The EGFR gene product is a transmembrane glycoprotein belonging to the epidermal growth factor receptor family and its cytoplasmic domain is responsible for sending the mitogenic signals into cells. We discovered that this domain of EGFR interacts with Tid1 protein, a human counterparts of Drosophila tumor suppressor Tid56. Tid56 null mutation causes lethal tumorigenesis during larvae stage. Tid1 is also known as a cochaperone of the heat shock protein 70 (Hsp70) and binds to Hsp70 through its conserved DnaJ-domain. We found that Increased expression of Tid1 in human carcinoma attenuates the EGFR-dependent oncogenic ERK1/2 and BMK1 signaling pathways. Importantly, the functional DnaJ-domain of Tid1 is required for consequent suppression of oncogenic signaling of carcinoma cells resulting from increased Tid1 expression. Together, these results suggest that Tid1 deterring controlled proliferation of carcinoma cells through reducing the downregulating the cancerous signaling from EGFR. Moreover, the cochaperonic and regulatory function of Tid1 on Hsp70 most likely play an essential role of this anti-proliferation function of Tid1 in carcinoma cells.				
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## INTRODUCTION

This study is focused on the neurotoxic actions of the insecticides permethrin (PM) and chlorpyrifos (CPF) as they relate to the development of Parkinson's Disease (PD). These compounds possess properties that could damage the nigro-striatal system, which is the primary brain lesion in PD (Bowman and Rand, 1980). We assessed the ability of each compound alone, or in combination, to directly induce neurochemical or neuropathological hallmarks of PD. In addition, tests with different isomers of PM attempted to identify those involved in its neurochemical effects on striatal dopaminergic systems. Since PD is hypothesized to have a multifactorial etiology (Siderowf, 2001), these insecticides are also being tested for their ability to synergize the actions of the established Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This approach will determine any ability of the insecticides to accelerate or intensify idiopathic disease processes. Experiments are performed on the C57BL6 black mouse, which when given MPTP is a valid rodent model for the development of PD (Heikkila and Sonsalla, 1992). For each treatment group, effects consistent with metabolic insult and changes in cholinergic and dopaminergic neurotoxicity in the striatum are measured. Cell stress in striatal nerve terminals is evaluated by measurements of mitochondrial function. Other neurochemical studies measured effects specific to the dopaminergic pathways in the striatum, including the amounts of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid, as well as the ability of isolated nerve terminals to transport dopamine. Because of the anticholinergic effect of chlorpyrifos, we also measure acetylcholinesterase activity, and the density/function of muscarinic and nicotinic receptors following insecticide treatment. Neuropathology studies are focused on any gross changes in immunocytochemical markers for glial fibrillary acid protein (GFAP). Other antibody labeling studies will assess effects specific to the dopaminergic system, including antibody labeling of tyrosine hydroxylase and the dopamine transporter (DAT), including identification of the PM isomer involved in its up-regulation. These studies represent a unique combination of research approaches and will provide a comprehensive and integrated evaluation of the possible Parkinsonian effects of these insecticides.

## BODY

This project has three main objectives: to establish the no observable effect level (NOEL) for insecticide exposures using the biomarkers listed below; to ascertain any synergistic interactions between the insecticides and the parkinsonian neurotoxin, MPTP; and finally, evaluate the reversibility of effects by assessing biomarkers at various times post-treatment. These objectives were pursued in retired breeder male (7-9 months old) C57 mice using the treatment regime shown in Fig. 1, with only a subset of selected doses and biomarkers proposed for objectives 2 and 3.

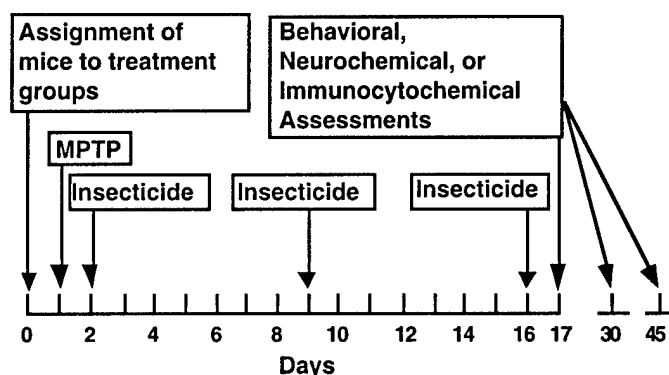


Figure 1. Treatment regime for studies of insecticides alone or in combination with MPTP. Treatment groups were: Controls, PM, CPF, MPTP, MPTP + PM, MPTP + CPF, PM + CPF, and MPTP + PM + CPF. Animals receiving MPTP alone were also given insecticide vehicles.

Supplemental funding for a single graduate student (Jinghong Kou) was provided by the sponsor to identify isomers of permethrin responsible for DAT upregulation. Nonetheless, a significant amount of time in 2003 was allocated to analysis of ongoing experiments. Results are organized by alphabetical listing of biomarker assessments, as given in the amended grant proposal and in the annual reports for previous years.

### Biomarker Findings for the Entire Grant Period:

- a. Assess toxicant effects on dopamine titers and turnover by measuring the dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC) content of the striata from treated mice.

No experiments of this type were run in the last year. In previous studies, we found at best small synergistic effects of insecticides with MPTP in measures of striatal dopamine content (see conclusions from reports for 2000 and 2001) at the highest dose of MPTP we used (30 mg/kg). However, all these measurements were performed at only one time point (1 day post-treatment). Related studies in the past two years used DAT expression as a dopaminergic biomarker in place of dopamine and DOPAC titers. This approach was used because western blot analysis can be performed on smaller treatment groups of mice than dopamine analysis by HPLC, and therefore conserved experimental animals.

- b. Assess effects on the density and kinetic properties of dopamine transporters in striatal synaptosomes from treated mice.

GBR binding, as well as Western blot analysis of DAT, TH,  $\alpha$ -synuclein, and synaptophysin was approached by the methods described in Gillette and Bloomquist (2003), which can be found in the appendix. This paper has data relevant to objectives 1 and 3 (NOEL determination and reversibility) for PM effects. We focused on the upregulation of the DAT because it could provide a molecular gateway for parkinsonian toxins to access dopaminergic systems (e.g., MPP<sup>+</sup>). A significant amount of effort in the past year was expended in completing ongoing studies of the levels of DAT protein expressed at different post-treatment times after 1.5 mg/kg PM (Fig. 2).

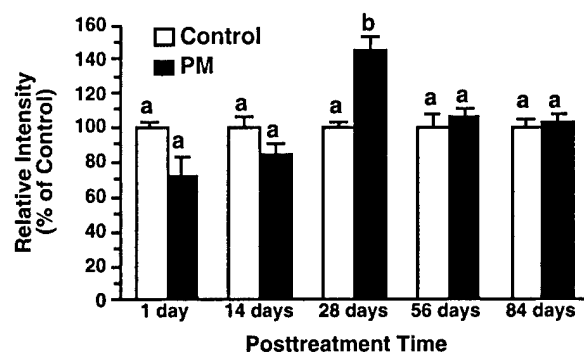


Fig. 2. Time course experiments of DAT western blots in C57Bl/6 mice treated with 1.5 mg/kg PM. Bars are means  $\pm$  SEM. Letters denote results of ANOVA on raw data with Student-Newman-Keuls means separation test, where treatment effects are significantly different ( $p < 0.05$ ) when labeled by different letters.

At this dose, we observed an upregulation of DAT expression, consistent with previous findings (Karen et al., 2001; Gillette and Bloomquist, 2003). This upregulation peaked 28 days post-treatment, and declined thereafter. These results demonstrate that this effect of PM can be slowly developing, and is reversible.

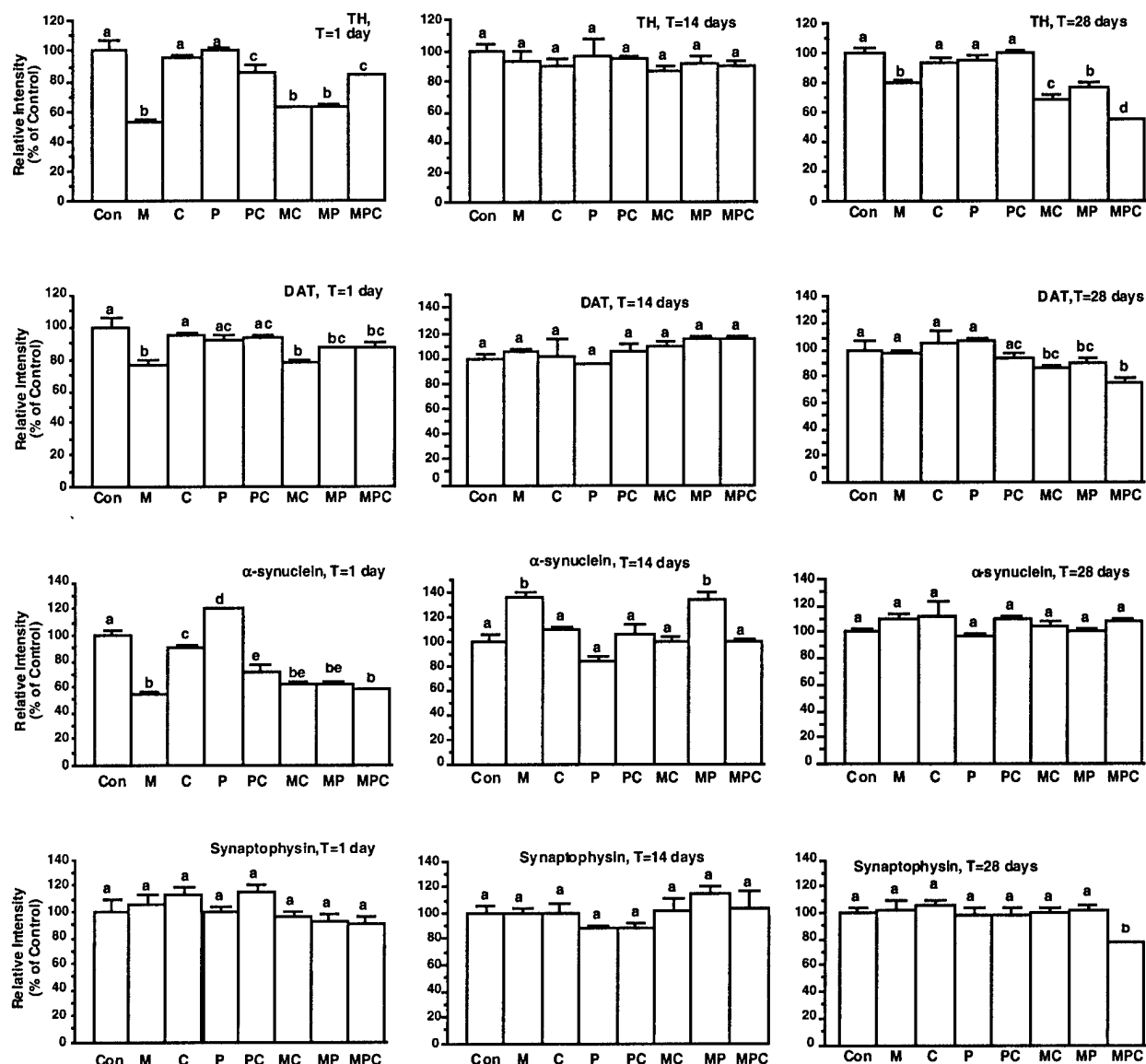


Fig. 3. Protein expression in combination treatments, assayed 1, 14, or 28 days after the last injection (Fig. 1). Bars are means  $\pm$  SEM. Treatments are as follows: Con=controls given methoxytriglycol (vehicle for permethrin), saline (vehicle for MPTP), and corn oil (vehicle for chlorpyrifos); M=MPTP, 20 mg/kg; C=chlorpyrifos, 75 mg/kg; P=permethrin, 200 mg/kg; PC=permethrin+chlorpyrifos; MC=MPTP+chlorpyrifos; MP=MPTP+permethrin; and MPC=MPTP+permethrin+chlorpyrifos. Other abbreviations are: DAT=dopamine transporter; TH=tyrosine hydroxylase. ANOVA was performed on the raw data before calculation as % of control, with Student-Newman-Keuls means separation test. Treatment effects are significantly different ( $p < 0.05$ ) when bars are not labeled by the same letter.

Other work in 2003 evaluated protein expression in combination treatment groups to investigate any additive or synergistic effects. These studies were related to all three major technical objectives of the project, where the T=1 day data is new and the T=14 and T=28 day data are corrected analyses of what we reported last year, using a manual method of delineating bands for image analysis. Densitometry scans of western blots (Fig. 4) showed a pattern indicative of the established effects

of MPTP, as well as a significant, slowly developing neurotoxicity when this compound was combined with insecticide treatments. Insecticides alone had little or no effect. Under exposures similar to the T=1 day group discussed here, Tillerson *et al.* (2003) found a decrease in both DAT and TH expression in western blots of striatal tissue induced by MPTP. In our studies, TH expression was decreased by MPTP, but not by insecticide treatments at T=1 day, with no effect 2 weeks later. By T=28 days post-treatment, there was a decline in TH levels, especially evident in the triple treatment group (Fig. 4). A similar pattern of effect through time was also observed in the expression of the DAT (Fig. 4). In contrast,  $\alpha$ -synuclein expression is suppressed by most treatments relative to controls at T=1 day. An exception is 200 mg/kg PM, which elevated  $\alpha$ -synuclein expression at this time, as reported previously (Gillette and Bloomquist, 2003). Except for the MPTP and MPTP+PM groups, which are elevated, all groups show normal levels of  $\alpha$ -synuclein by T=14 days, with control levels of expression occurring in all groups at T=28 days (Fig. 4). The decline, increase, and return to normal levels of  $\alpha$ -synuclein expression by MPTP (Fig. 4) is roughly similar to the results of Vila *et al.* (2000). They observed that chronic exposure to MPTP (30 mg/kg a day for 5 days to 8 week old C57 mice) caused about 50% increase in  $\alpha$ -synuclein 0-4 days post-treatment, but levels had returned to normal by day 7. In our studies, there was no effect on synaptophysin levels in any group tested, except for the triple treatment group at T=28 days, consistent with the delayed neurotoxicity observed for this group with respect to DAT and TH expression (Fig. 4). Vila *et al.* (2000) found that chronic exposure to MPTP as described above caused about a 50% reduction in synaptophysin that persisted to post-treatment day 7. The single dose of 20 mg/kg MPTP we used in the present study may account for the lack of effect on synaptophysin expression when it was applied alone. The slow time course and persistence of the effect on TH, DAT, and synaptophysin suggests that the combination exposure to all three compounds is setting in motion a slowly developing neurotoxicity. These findings suggests that insecticide exposure may exacerbate idiopathic disease processes.

**c. Compare the extent of toxin-dependent actions on mitochondrial function in striatal synaptosomes by measuring thiazolyl blue (MTT) dehydrogenase activity.**

No experiments of this type were run in the last year.

**d. Search for anatomical evidence of general neurotoxicity within light microscopic preparations of the nigro-striatal system by examining glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for gliosis.**

**e. Search for anatomical evidence of general neurotoxicity within specific dopaminergic components of the nigro-striatal system using immunocytochemical staining for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH).**

**f. Confirm whether functional changes in dopamine transport are due to fluctuating levels of dopamine transporter (DAT) protein using immunocytochemical identification.**

As in past reports, these biomarkers are dealt with together since fixation, sectioning, staining and analysis of the tissue are similar for each. The methodology employed in these studies can be found in the appended publication (Pittman *et al.*, 2003, in the appendix). In this study, the major findings were a significant decrease in DAT expression at 3 mg/kg PM (no effect at 0.8 and 1.5 mg/kg), and an increase in GFAP at 200 mg/kg PM (no effect on TH or DAT at this dose). All these studies were done 24 hr after the last treatment, and are generally consistent with our western blot studies. We speculate that the lack of effect on DAT expression may be a difference in tissue sub-sampling, which is discussed at length in Pittman *et al.* (2003).

Three additional experiments in the past year were designed to assess possible synergistic effects of 1) permethrin, 2) chlorpyrifos and 3) permethrin combined with chlorpyrifos upon MPTP neurotoxicity. For this work, pixel counts for tissue from all experimental conditions were performed as noted in Pittman *et al.* (2003). However, for each of the three experiments, the design was a 2 x 2 factorial with 16 mice assigned to each of the 4 treatment groups: vehicle control, pesticide alone, MPTP alone, or both pesticide and MPTP. Within a given experiment, matched sets of 4 mice, one from each treatment group, were dosed, sacrificed and histologically processed together and referred to as a 'case'. For each case, three tissue slides were prepared for TH staining: a rostral striatal slide taken 1410  $\mu$ m caudal to the rostral tip of the striatum, a caudal striatal slide taken 2820  $\mu$ m caudal to the rostral tip of the striatum, and an omission control slide taken immediately adjacent to the rostral striatal location. Three additional slides, from the same regions, were prepared for GFAP staining, except that the omission control slide was taken from the caudal striatal location. Each microscope slide had one brain slice from each of the 4 mice comprising a case, and thus had one slice from each of the four treatment groups (vehicle control, pesticide alone, MPTP alone or both pesticide and MPTP). Mice were dosed with either MPTP or vehicle on Day 1, and with either pesticide or vehicle on Days 2, 9, and 16, and sacrificed on Day 17. The TH antibodies used in this experiment, from Pel-Freeze, were used at a dilution of 1:400; the GFAP antibodies, from Chemicon International, were used at a dilution of 1:3000.

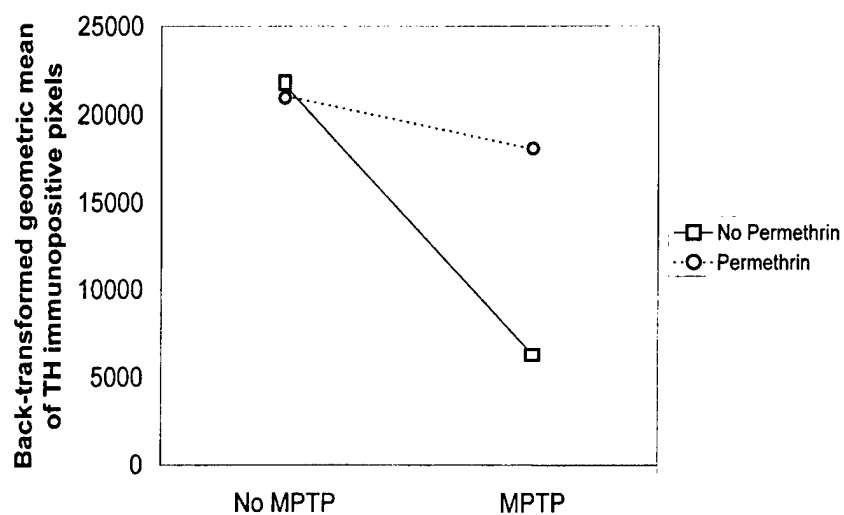
For analysis of the tissue in the MPTP synergy experiments, initial scatterplots of residual error were examined to assess the viability of the model used for analysis. This suggested that a log transformation should be used on the data. Therefore, all reported values are geometric means that have been back-transformed from their log values. Within each experiment, the rostral and caudal striatal locations, for a given tissue stain, were run as separate analyses. A two-way ANOVA was run for each analysis, using SAS (SAS Institute Inc., Cary, NC, USA), and looked at the main effect of pesticide, the main effect of MPTP and the pesticide/MPTP interaction. We have not yet had a chance to analyze the changes in GFAP immunoreactivity in the corpus callosum for these MPTP synergy experiments and, therefore, we are not yet sure of their striatal-specific nature. Furthermore, although a log transform significantly improved the distribution of our residual error, we feel that an alternative model may further improve the sensitivity of our statistical analysis and we are currently exploring the use of that model.

We have recently completed our analysis of TH and GFAP immunoreactivity in mice exposed to 200 mg/kg of permethrin combined with 30 mg/kg of MPTP. The experimental design and statistical analysis were described above. For each immunohistochemical stain, two rostrocaudal locations were examined in the striatum. At both locations, there was a significant main effect decrease in TH immunostaining produced by MPTP. Using the back-transformed geometric means, this decrease was 50.2% ( $df = 1,39$ ;  $p = 0.035$ ) at the rostral location and 66.6% ( $df = 1,37$ ;  $p = 0.0003$ ) at the caudal location in groups receiving MPTP compared with groups not receiving MPTP. However, there was no significant main effect of permethrin upon TH immunostaining, nor was there a significant interaction between the effects of permethrin and MPTP.

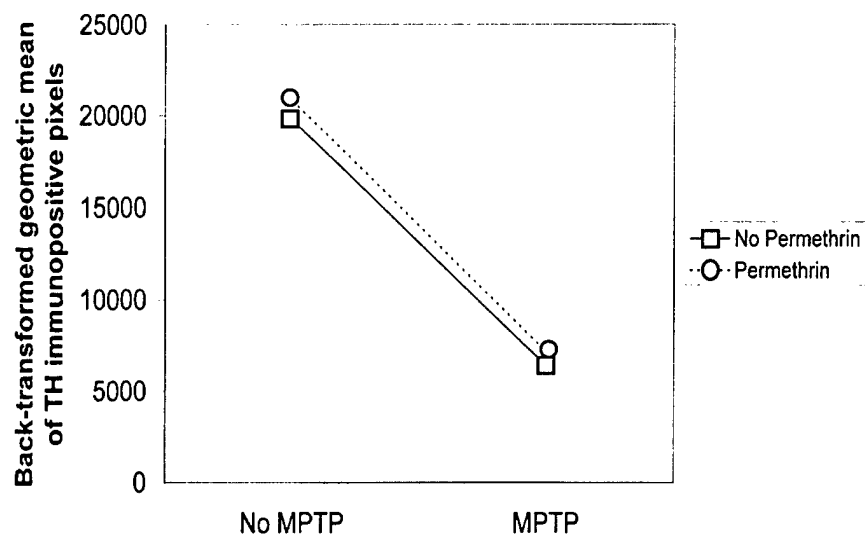
For GFAP, there was a significant main effect increase in immunostaining in groups treated with MPTP compared with groups not treated with MPTP, at both rostrocaudal locations in the striatum. This increase was 6,017% ( $df = 1,42$ ;  $p < 0.0001$ ) at the rostral location and 1,996% ( $df = 1,38$ ;  $p < 0.0001$ ) at the caudal location. As mentioned earlier, there was also a significant main effect increase in GFAP in the permethrin treated groups at both striatal locations. The increase was 90.8% ( $df = 1,42$ ;  $p = 0.03$ ) at the rostral location and 120.9% ( $df = 1,38$ ;  $p = 0.03$ ) at the caudal location. There was no significant interaction between the effects of permethrin and MPTP upon GFAP immunostaining in the striatum. The back-transformed geometric means for GFAP and TH immunoreactivity, calculated from the log values used in the statistical analysis, can be seen in Figure 4. Since permethrin produced the only significant insecticide-induced main effect in our MPTP synergy experiments, while the effect of MPTP was fairly similar across these experiments, a figure is only presented for the permethrin/MPTP experiment.



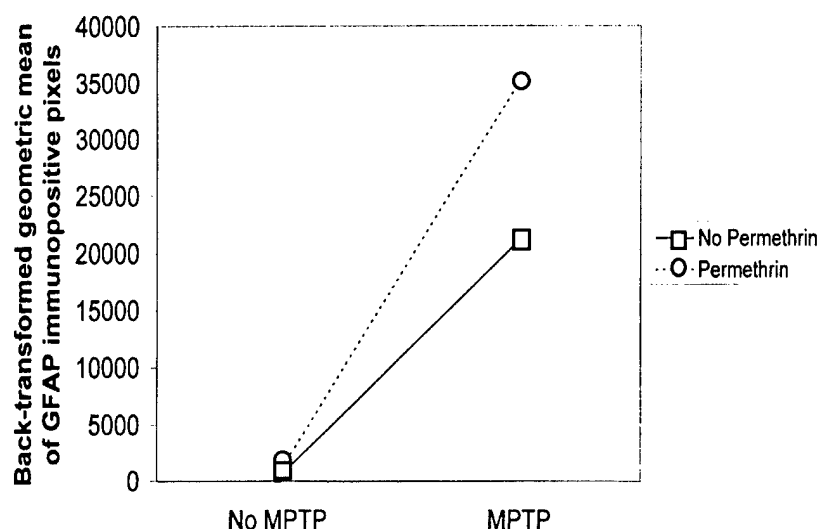
**A. 200 mg/kg Permethrin + 30 mg/kg MPTP**  
**Rostral Striatum: TH**



**B. 200 mg/kg Permethrin + 30 mg/kg MPTP**  
**Caudal Striatum: TH**



**C. 200 mg/kg Permethrin + 30 mg/kg MPTP**  
**Rostral Striatum: GFAP**



**D. 200 mg/kg Permethrin + 30 mg/kg MPTP**  
**Caudal Striatum: GFAP**

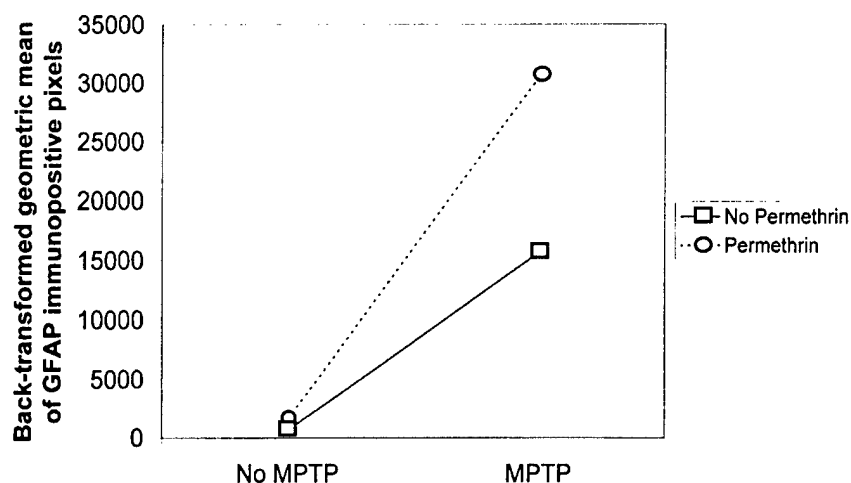


Figure 4. Back-transformed geometric means, calculated from the log values used in the ANOVA, illustrating the effects of permethrin (200 mg/kg) and MPTP (30 mg/kg) in mice dosed with both compounds. Means are presented for TH (A & B) and GFAP (C & D) immunoreactivity in both the rostral (A & C) and caudal (B & D) striatal locations.

An experiment with the identical design and statistical analysis to that just described was performed to examine synergistic interactions between chlorpyrifos and MPTP in mice dosed with 50 mg/kg chlorpyrifos and 30 mg/kg of MPTP. Although there was a 47.1% reduction in TH immunostaining for MPTP-treated groups compared with non-MPTP treated groups, this change was not significant at the rostral striatal location. However, at the caudal location, there was a

significant 53.2% ( $df = 1,44$ ;  $p = 0.030$ ) main effect reduction for the MPTP treated groups. As noted earlier, there was no significant main effect of chlorpyrifos at either striatal location. There was also no significant interaction of chlorpyrifos and MPTP upon TH immunostaining in the striatum.

With regard to GFAP, there was a significant main effect increase in immunostaining in groups treated with MPTP compared with groups not treated with MPTP, at both rostrocaudal locations in the striatum. This increase was 3,619% ( $df = 1,43$ ;  $p < 0.0001$ ) at the rostral location and 1,142% ( $df = 1, 44$ ;  $p < 0.0001$ ) at the caudal location. However, there was no significant main effect of chlorpyrifos upon GFAP immunostaining at either striatal location, nor was there a significant interaction between chlorpyrifos and MPTP.

The same experimental strategy was used to examine synergistic interactions between a combined 200 mg/kg permethrin + 50 mg/kg chlorpyrifos dosing and a 30 mg/kg dosing of MPTP. At the rostral striatal location, there was a significant 69.9% ( $df = 1,42$ ;  $p = 0.003$ ) main effect decrease in TH immunostaining produced in MPTP-treated versus non-MPTP-treated groups. However, the 39.7% decrease in MPTP-treated groups observed at the caudal striatal location was not significant. Furthermore, there was no significant main effect of the combined pesticide dose upon TH immunoreactivity at either striatal location, nor was there a pesticide-MPTP interaction at either location.

As for the previous two experiments, there was a significant main effect increase in GFAP immunostaining in groups treated with MPTP compared with groups not treated with MPTP, at both rostrocaudal locations in the striatum. This increase was 7,973% ( $df = 1,43$ ;  $p < 0.0001$ ) at the rostral location and 1,117% ( $df = 1, 44$ ;  $p < 0.0001$ ) at the caudal location. However, there was no significant main effect of permethrin + chlorpyrifos upon GFAP immunostaining at either striatal location, nor was there a significant interaction between the combined pesticide dose and MPTP.

Our immunohistochemical MPTP synergy experiments were fairly consistent in demonstrating an expected decrease in TH immunoreactivity and an increase in GFAP immunoreactivity in the striatum in MPTP treated groups. This result would be expected from the degeneration of nigrostriatal terminal neuropil. However, unlike the western blot results of Fig. 3, there was no evidence that permethrin and chlorpyrifos, alone or together, could alter the effects upon TH or GFAP immunoreactivity produced by MPTP. This difference is most likely related to the fact that the immunocytochemical analyses were performed at T=1 day, whereas the western blots showed the greatest effect at T=28 days (Fig. 3).

**g. Explore toxicant effects on open field/rearing frequencies and pole climbing behaviors and search for correlations between behavioral impairment and neurochemical effects.**

Methods for measuring rearing and movement frequencies in treated mice can be found in Karen et al. (2001) or previous reports. Behavioral experiments focused on measurements of impairment of motor function. Alteration of motor behaviors was assessed using open field ambulation, rearing frequency, and a pole traction test similar to those described by Takahashi *et al.* (1989). Animals were tested at the end of the post-treatment period, prior to sacrifice for the neurochemical analyses. In the open field test, we assigned a movement unit whenever all 4 feet of the mouse entered a new square on the bottom of the arena. Movement units and the number of rearing behaviors produced were summed over a 3 min observation period. The number of rears was also summed for 3 min. The pole test apparatus consists of a 38 cm taped ring stand. Mice are placed at the top of the pole and allowed to hang by the forepaws. All mice are observed for up to 5 minutes. Times required for mice to turn (invert) and then climb down the pole are measured. Inversion times, climbing times, and the number of falls was determined.

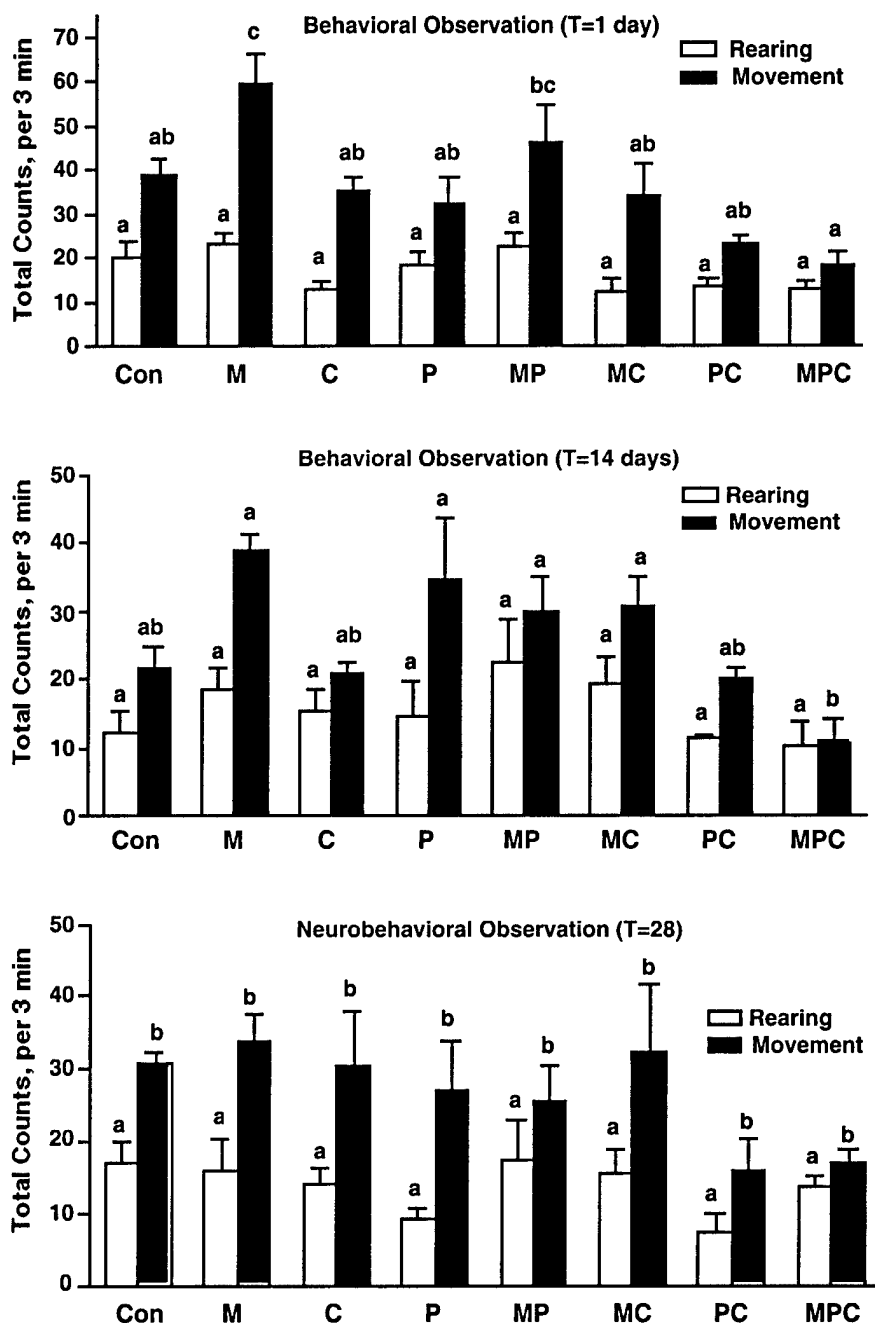


Figure 5. Movement and rearing frequencies in C57 mice treated with toxicants, assayed at three times (1, 14, or 28 days) following the last injection. Bars are means  $\pm$  SEM. Treatments are as follows: Con = controls given methoxytriglycol (vehicle for permethrin), saline (vehicle for MPTP), and corn oil (vehicle for chlorpyrifos); M=MPTP, 20 mg/kg; C=chlorpyrifos, 75 mg/kg; P=permethrin, 200 mg/kg; PC=permethrin + chlorpyrifos; MC=MPTP+chlorpyrifos; MP=MPTP+permethrin; and MPC=MPTP+permethrin+chlorpyrifos. ANOVA was performed for each behavioral parameter, followed by Student-Newman-Keuls means separation test. Treatment effects within a given behavioral parameter are significantly different ( $p < 0.05$ ) when not labeled by the same letter.

The data shown in Fig. 5 are for the same mice whose neurochemical analyses were shown in Fig. 3. The main finding at T=1 day post-treatment is a significant increase in movement by MPTP.

This is the second time we have observed this paradoxical effect, perhaps due to the single, low dose of MPTP we used. There is a clear trend for decreasing movement across all three time points in the combination treatments, especially the triple treatment group (MPTP+PM+CPF). However, there were few statistically significant effects when the data were analyzed as a single analysis set, perhaps because of the small sample size ( $n = 5$  mice/group). In fact, ANOVA of the rearing data at T=1 day found statistical significance among the treatment effects ( $p = 0.0055$ ), but Student-Newman-Keuls, Bonferroni, or Tukey's post tests failed to find significance among the treatment means. This situation is documented in the statistical literature (Zar, 1984), and arises because ANOVA is more powerful than the post tests mentioned above. We are initially limited to these three post tests because they are the ones available on InStat™ (GraphPad software, San Diego, CA), the software we use for data analysis on a day to day basis. We will seek advice from the Dept. of Statistics here at Virginia Tech on how to proceed, and modify our analyses accordingly. The reduction in behavior correlates with the data of Fig. 3, where western blot analysis for DAT, TH, and synaptophysin were all decreased significantly in the triple treatment group. No clear effect on pole traction behavior was observed in this experiment (data not shown).

**h. Determine the extent of acetylcholinesterase inhibition following treatment with toxicants for comparison with other behavioral and neurochemical effects.**

No experiments of this type were run in the last year.

**i. Define any toxicant-induced changes in cholinergic receptor density or function with respect to agonist-induced dopamine release from striatal synaptosomes.**

Although no experiments of this type were run in the last year, we have frozen tissue samples from the synergism experiments of Fig. 3 that are sufficient to determine QNB and epibatidine binding studies (single saturating concentration) in cortical and striatal membranes at T=1, 14, and 28 days post-treatment.

We have not yet performed any studies on the ability of cholinergic compounds (nicotine or muscarine) to alter release of dopamine in striatal synaptosomes from insecticide-treated mice, although we have performed preliminary studies to confirm our technical ability to do so.

**j. Define which isomer of PM is responsible for the upregulation of the dopamine transporter.**

PM has two chiral carbons in the acid moiety, and exists as four diastereomers. We used the treatment regime of Fig. 1 for these studies and purified samples of *cis*-PM and *trans*-PM to begin this work. The results are shown in Fig. 6. There were no significant effects of any of the treatments, even the technical material, which has upregulated the DAT and  $\alpha$ -synuclein in the past (Gillette and Bloomquist, 2003). The effect of technical PM on  $\alpha$ -synuclein at T=1 day is almost statistically significant, considering that the controls were only replicated twice, but the effect in these experiments were much smaller in magnitude than the ca. 70% up-regulation we observed previously for  $\alpha$ -synuclein (Gillette and Bloomquist, 2003). The present studies were performed by Ms. Jinghong Kou, a graduate student, who joined this project in 2002, and worked closely with Dr. Jeff Gillette. This past year, she has been working on her own, and her relative inexperience may have contributed to problems of replicability. We are confident of the PM-induced upregulation of the DAT, since this effect was repeatedly observed by two postdocs running dopamine uptake, GBR binding, and western blot analyses at different times on different cohorts of mice (Karen et al., 2001; Bloomquist et al., 2002; Gillette and Bloomquist, 2003). The lack of significant effect of the positive controls, obviated the study of enantiomerically pure isomers. We are currently trying to identify the technical problem with these experiments.

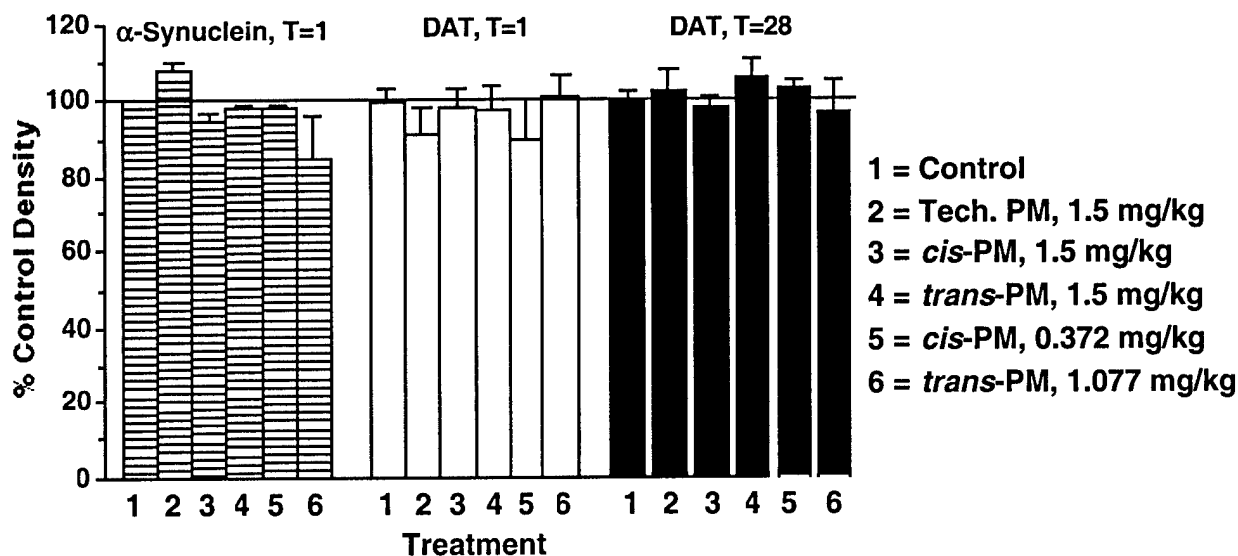


Figure 6. Effects of PM isomer on DAT and  $\alpha$ -synuclein expression analyzed by western blot. ANOVA found no significant treatment effects. PM was tested at 1.5 mg/kg for the technical (Tech.), *cis*, and *trans* materials. The doses of 0.372 mg/kg (*cis*) and 1.077 mg/kg (*trans*) reflect the proportion of each isomer in the technical product (24.8% *cis* and 71.8% *trans*, with overall purity of 96.6%)

## KEY RESEARCH ACCOMPLISHMENTS

Demonstrated a slowly developing enhancement of MPTP toxicity by insecticides (PM and/or CPF) occurring at least four weeks after treatment (Fig. 3).

The greater neurochemical effects observed at T=28 days post-treatment correlate with a trend for greater behavioral dysfunction at this time (Fig. 5).

Upregulation of the DAT by PM is slowly developing, but eventually reverses during prolonged post-treatment observation times (Fig. 2).

## REPORTABLE OUTCOMES FOR 2003

### Meeting Presentations (speaker underlined>

none

### Publications in 2003

J. S. Gillette and J. R. Bloomquist, Differential Up-Regulation of Striatal Dopamine Transporter and  $\alpha$ -Synuclein by the Pyrethroid Insecticide Permethrin. *Toxicol. Appl. Pharmacol.* 192, 287-293 (2003).

J. Pittman, C. Dodd, and B. Klein, Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin. *Intl. J. Toxicol.* 22, 359-370 (2003). Figure 1 from this paper was selected as cover art for this issue of the Journal (see appendix).

Writing of a paper on dopaminergic biomarkers (e.g., Fig. 3), along with previous dopamine depletion data will commence soon. Submission of a paper on cholinergic markers awaits the addition of synergism data at T=1 and T=28 days for QNB and epibatidine binding. Dr. Klein will submit a paper on the interactions of insecticides with MPTP in immunocytochemical studies as well. We hope to complete all of these papers by the end of 2004.

## CONCLUSIONS FOR 2003

The up-regulation of dopamine transport occurring at low doses of PM (Gillette and Bloomquist, 2003) provides a mechanism for possible synergism with pyridinium toxins, such as MPP<sup>+</sup>. The enhanced effect of PM, CPF, and MPTP in T=28 day analyses of DAT, TH, and synaptophysin expression suggests that insecticide exposure could induce or exacerbate idiopathic disease processes. However, we have so far only demonstrated synergism at high doses of insecticides.

Upregulation of the DAT and  $\alpha$ -synuclein may provide a mechanism for pyrethroid-induced neurodegeneration. It has been reported that human  $\alpha$ -synuclein has the ability to complex with human DAT, causing acceleration of cellular DA uptake and DA-induced cellular apoptosis (Lee et al., 2001). DA itself can be metabolized to toxic free radical species that may have neurotoxic effects, including inhibition of mitochondrial respiration (Ben-Shachar et al., 1995). Thus, the combined increase in DAT and  $\alpha$ -synuclein by pesticides such as PM may exacerbate this potential for cellular toxicity, especially if sufficiently prolonged. Our findings with  $\alpha$ -synuclein, while pertinent to these studies, are not part of our plan of work for this project.

We plan to pursue longer term exposures (weekly treatments over 3 and 6 months) in a recently awarded Supplement to this project.

GFAP expression seems to be a more sensitive indicator of toxic insult than TH levels in immunocytochemical studies, and although each was effective alone, there was no significant enhancement of effect when combining high doses of PM with MPTP. Differences in results between western blot and immunocytochemical studies may be due to sampling effects.

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## APPENDICES

### Published Papers

See attachments.



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## Differential up-regulation of striatal dopamine transporter and $\alpha$ -synuclein by the pyrethroid insecticide permethrin

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### Abstract

The effects of permethrin on striatal dopaminergic biomarkers were assessed in this study. Retired breeder male C57 B1/6 mice were given an ip dose of permethrin (0.1–200 mg/kg) at 7-day intervals, over a 2-week period (Days 0, 7, and 14). Animals were then sacrificed 1 day ( $t = 1$ ), 14 days ( $t = 14$ ), or 28 days after the last treatment ( $t = 28$ ). Dopamine transporter (DAT) protein as assayed by Western blotting was increased to 115% in the 0.8 mg/kg group over that of control mice at  $t = 1$  ( $P < 0.05$ ). At  $t = 14$ , this value increased to 140% of control, and declined slightly to 133% of control at  $t = 28$ . The mice given the 1.5 mg/kg dose displayed a significant increase in DAT protein only at  $t = 28$ , to 145% of controls. Thus, upregulation of the DAT at low doses of PM is variable 24 h after treatment, and seems to stabilize by  $t = 28$ . The threshold dose for increasing DAT expression in Western blots by  $t = 28$  was 0.2 mg/kg permethrin. [ $^3\text{H}$ ]GBR 12935, used to assay DAT binding, followed the same trend as that for the Western blotting data for 0.8 and 1.5 mg/kg doses of permethrin over the 4 weeks posttreatment. At 200 mg/kg permethrin, DAT protein was unchanged vs controls ( $t = 1$ ), but had significantly increased by  $t = 14$  and continued to increase at  $t = 28$ , suggesting that the reduced dopamine transport at this dose was due to nerve terminal stress and that recovery had occurred. The protein  $\alpha$ -synuclein was also significantly induced at the 1.5 mg/kg dose at  $t = 1$ ; however, unlike DAT up-regulation, this effect had declined to control values by  $t = 14$ . Maximal induction of  $\alpha$ -synuclein protein occurred at a dose of 50 mg/kg permethrin. These data provide evidence that the pyrethroid class of insecticides can modulate the dopaminergic system at low doses, in a persistent manner, which may render neurons more vulnerable to toxicant injury.

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**Keywords:** Parkinson's disease; Striatum; Neurotoxicity; Protein regulation

### Introduction

Parkinson's disease (PD), a chronic neurodegenerative disease of unknown etiology, is characterized by a loss of dopaminergic neurons in the substantia nigra and depletion of striatal dopamine (DA) (Fearnley and Lees, 1991). In humans, an 80% striatal DA loss is required before overt clinical symptoms appear, after which significant correlation exists between severity of PD symptoms and subsequent DA loss (Hornykiewicz and Kish, 1986).

The etiology of idiopathic PD is thought to be multifactorial (Paganini-Hill, 2001; Siderowf, 2001; Zhang et al., 2000), and it has long been known that there is an epide-

miological link between PD and persons who are associated with rural living and agricultural work. In particular, persons exposed to various herbicides and insecticides in an agricultural setting show an increased risk of developing PD (Semchuck et al., 1992, 1993; Gorell et al., 1998). Other work linked exposure to organochlorine insecticides such as dieldrin to PD (Fleming et al., 1994). The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is able to produce parkinsonism in animals and humans via its oxidative metabolite MPP<sup>+</sup> (Tipton and Singer, 1993) provided the original evidence for a putative environmental component in the etiology of PD (Jenner et al., 1992).

The so-called Gulf War Syndrome, which is manifest as various neurological maladies reported by veterans of the Persian Gulf War, may be linked to pesticide exposure. These compounds include pyridostigmine bromide (PB), an

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anticholinesterase, the insect repellent DEET, and the pyrethroid insecticide permethrin (PM), which was impregnated into the uniforms of soldiers who served in the war (Hoy et al., 2000). Personnel who served in the first Gulf War report a variety of symptoms of neurological dysfunction, with no mortality increases, which have been confirmed by epidemiological studies (Steele, 2001). Further, these symptoms may be related to a spectrum of generalized neurologic injury to the central, peripheral, and autonomic nervous systems (Haley et al., 1997a, 1997b).

The dopamine transporter (DAT) is a membrane-bound carrier molecule that mediates the action of DA in the nerve synapse via the reuptake of DA into the dopaminergic neuron, and the DAT is also capable of neuronal DA release (Horn, 1990). In addition, MPP<sup>+</sup> is transported into the neuron via the DAT, where it blocks mitochondrial complex I respiration and subsequently causes cell death (Tipton and Singer, 1993). Thus, the DAT is a putative molecular gateway for exogenous and endogenous dopaminergic toxicants.

Using three ip doses over a 2-week period (Days 0, 7, and 14), our laboratory found that the organochlorine insecticide heptachlor has significant effects on DA transport and causes DAT up-regulation in C57B1/6 mice at relatively low doses (Miller et al., 1999; Kirby et al., 2000). We also showed that the pyrethroid insecticides deltamethrin and PM significantly increase DA uptake in treated mice (Kirby et al., 1999; Karen et al., 2001). For PM, DA uptake was increased 33% above controls ( $P < 0.05$ ) at a dose of 1.5 mg/kg, but reduced 56% below control values ( $P < 0.05$ ) at 200 mg/kg (Karen et al., 2001). The objective of this research was to further characterize the time and dose dependence of PM's effects on transporter ligand binding and DAT protein expression. Moreover, because mutations in the  $\alpha$ -synuclein gene are associated with some forms of familial PD (Langston et al., 1998; Mizuno et al., 2001), and the herbicide paraquat causes up-regulation and increased aggregation of  $\alpha$ -synuclein in C57B1/6 mice (Manning-Bog et al., 2001), we have expanded our studies to include Western blot analysis of  $\alpha$ -synuclein expression in PM-treated mice. In this way, we hope to gain insight into the mechanisms by which compounds alter the dopaminergic system and render the neuron more susceptible to toxicant injury.

## Materials and methods

**Chemicals.** Technical PM (mixture of 1-*R,S*-*cis* and 1-*R,S*-*trans* isomers) was purchased from Sigma-Aldrich GmbH. Sucrose, Hepes, KCl, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Methoxytriglycol (MTG), NaCl, CaCl<sub>2</sub>, MgSO<sub>4</sub>, and Ponceau-S stain were purchased from Sigma Chemical Co. (St. Louis, MO). GBR 12909 was obtained from Research Biochemicals International (Natick, MA), and [<sup>3</sup>H]GBR 12935 was obtained from NEN Life

Science Products, Inc. (Boston, MA). Acrylamide, bisacrylamide, ammonium persulfate, *N,N,N,N'*-tetramethylethylenediamine (TEMED), and Tris/glycine/SDS buffer were purchased from Bio-Rad (Hercules, CA). ECL Western blotting detection system RPN 2108 and ECL Hyperfilm were purchased from Amersham-Pharmacia Biotech, (Buckinghamshire, UK).

**Animals and treatments.** Retired breeder male C57B1/6 mice (Charles River Labs, Raleigh, NC) were weighed and segregated into randomized dosing groups ( $n = 6$  mice per dose group) prior to ip treatment with PM at doses ranging from 0.1 to 200 mg/kg. Mouse weights ranged from 35 to 40 g, with an average weight of 38 g. Different cohorts of mice were used for the low (0.2–1.5 mg/kg) vs high (100 and 200 mg/kg) PM dose groups. Further, the time course experiments were repeated using different cohorts of mice. For dose-response studies, a dose of PM dissolved in MTG was given three times over a 2-week period (Days 0, 7, and 14) and sacrificed 1 day ( $t = 1$ ) after the last dose as previously outlined in Kirby et al. (1999). This exposure paradigm is based on the results of Bloomquist et al. (1999), where a single treatment of MPTP caused significant depletion of striatal DA 2 weeks later. Control mice received MTG vehicle alone. For the time course studies, groups of mice were held for either 14 days ( $t = 14$ ) or 28 days ( $t = 28$ ) post last dosing, at which time they were sacrificed. At the time of necropsy, brain striatal tissues were dissected from the mice and prepared for assay as described below. All procedures were approved by the VPI&SU Animal Care and Use Committee.

**[<sup>3</sup>H]GBR 12935-binding assay.** Pooled striatal tissues from all mice within a treatment group were homogenized in ice-cold Krebs-Ringer's-Hepes (KRH) buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 20 mM Hepes, pH 7.5). Striata were then centrifuged at 18,000g and resuspended for 1 h in distilled water. The tissues were again centrifuged at 18,000g, resuspended in cold KRH buffer, and stored at  $-70^{\circ}\text{C}$  until use. For the assay, samples were thawed and run in duplicate in the absence and presence of 0.5  $\mu\text{M}$  GBR 12909. To measure total binding, [<sup>3</sup>H]GBR 12935 (100, 50, 25, 12.5, and 6.25 nM) was incubated with KRH buffer (20 mM Hepes, 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>; pH 7.5) and tissue for 2 h at  $4^{\circ}\text{C}$ . The same procedure was used to measure nonspecific binding with the exception that excess cold GBR 12909 was added to the reaction mixture. The reactions were stopped by the addition of cold KRH buffer, and the contents of each reaction tube were filtered at 10 psi using 25 mm Whatman GF/B filters presoaked in 0.1% BSA. Filters were then washed 3 times with cold KRH buffer to remove unbound radioactivity. The filters were soaked overnight in Scintiverse E, and total counts were measured on a Beckman LS 6500 liquid scintillation counter. Aliquots of [<sup>3</sup>H]GBR 12935 solutions were added

to Scintiverse E cocktail to calculate the exact [ $^3\text{H}$ ]GBR 12935 concentrations in each reaction mixture. Following protein determination by the method of Bradford (1976), nonlinear regression to isotherm plots was used to determine  $B_{\text{max}}$ ,  $K_d$ , and their standard errors using Prism (GraphPad Software, San Diego, CA).

**Western blot analysis.** Pooled striatal synaptosomes from C57B1/6 mice were homogenized in cold isolation buffer and centrifuged at 1000g for 15 min. The supernatant was removed and centrifuged at 10,000g for 15 min. The resulting pellets were resuspended in a small volume of KRH buffer and stored at  $-70^\circ\text{C}$  until use. At the time of assay, the tissues were thawed, mixed with sample buffer (60 mM Tris, pH 6.8, 2% SDS, 100 mM DTT, 0.001% bromphenol blue), and heated at  $80^\circ\text{C}$  for 5 min. Samples were run on either a 10% SDS gel (DAT, synaptophysin) or a 12% SDS gel ( $\alpha$ -synuclein) and transferred to a nitrocellulose membrane according to the method of Towbin et al., (1979). For the DAT,  $\alpha$ -synuclein, and synaptophysin blots, SDS gels were loaded with 10, 5, and 2  $\mu\text{g}$  of protein, respectively. After transfer was complete, the membrane proteins were stained with Ponceau S dye to verify transfer. The membrane was incubated overnight in 5% dry milk at  $5^\circ\text{C}$ , followed by an overnight incubation at  $5^\circ\text{C}$  in either a mouse monoclonal anti- $\alpha$ -synuclein (Bioscience International, Saco, ME), a rat monoclonal anti-DAT, or a rat monoclonal anti-synaptophysin primary antibody (Chemicon Int'l, Temecula, CA). The blot was washed with TBST buffer and incubated in an appropriate peroxidase-linked secondary antibody (Sigma Chemical Co.) for 1 h at room temperature. After a second washing with TBST buffer, the blot was developed using the ECL Amersham chemiluminescence kit (Amersham-Pharmacia Corp.), and exposed to ECL Hyperfilm (Amersham-Pharmacia Corp.) for varying lengths of time. Protein bands on the film were quantitated using the Kodak EDAS 290 system (Eastman Kodak Co.). Protein content was determined by the method of Bradford (1976). Gels were run with 3 protein samples (determinations) from the pooled tissue of each treatment group, and were quantitated by densitometry and then averaged. All blots contained matched controls, to account for any differences in staining or photographic development.

**Statistical analysis.** Statistical significance was determined using one-way ANOVA and Student-Newman-Keuls means separation if a statistically significant effect of treatment was observed. Other statistical comparisons were by  $t$  test calculations. Analysis was performed using InStat (GraphPad Software).

## Results

Signs of pyrethroid toxicity were not observed in any of the treatment groups, even at the highest dose of 200 mg/kg.

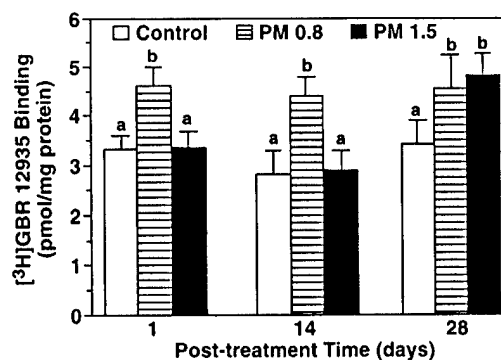


Fig. 1. Time course experiments of [ $^3\text{H}$ ]GBR 12935 binding in C57B1/6 mice treated with 0.8 or 1.5 mg/kg PM. Bars represent means with SEM in this and all subsequent figures. Letters denote results of ANOVA with Student-Newman-Keuls means separation test, where treatments within a particular time period (1, 14, or 28 days) are significantly different ( $P < 0.05$ ) when labeled by different letters.

Mouse weights were also not significantly altered by PM treatment in any of the dose groups (data not shown).

Since maximal DA uptake occurred at 1.5 mg/kg PM in our earlier DA uptake experiments (Karen et al., 2001), it was decided to select the PM doses of 0.8 and 1.5 mg/kg for the initial [ $^3\text{H}$ ]GBR 12935-binding assays and Western blotting studies. At  $t = 1$  day posttreatment, the  $B_{\text{max}}$  value for [ $^3\text{H}$ ]GBR 12935 was significantly elevated in the 0.8 mg/kg dose group to 137% of control, but was unchanged in the 1.5 mg/kg dose group (Fig. 1). At  $t = 1$  day,  $K_d$  values (nM,  $\pm$ SE) for the controls and 0.8 and 1.5 mg/kg groups were  $2.7 \pm 0.7$ ,  $3.9 \pm 0.9$ , and  $3.4 \pm 0.6$ , respectively. Other groups of mice from the same experimental cohort were assessed either 14 or 28 days beyond the last dose, with no further treatments given. At  $t = 14$  days, GBR 12935  $B_{\text{max}}$  increased to 157% of control in the 0.8 mg/kg dose group, with the 1.5 mg/kg dose group remaining unchanged. At  $t = 14$  days,  $K_d$  values (nM,  $\pm$ SE) for the controls and 0.8 and 1.5 mg/kg groups were  $3.4 \pm 1.9$ ,  $4.3 \pm 1.2$ , and  $6.0 \pm 1.8$ , respectively. At  $t = 28$  days, both the 0.8 and 1.5 mg/kg groups demonstrated a significantly increased [ $^3\text{H}$ ]GBR 12935  $B_{\text{max}}$  ( $\sim 130\%$  for both groups) over that of controls (Fig. 1). At  $t = 28$  days,  $K_d$  values (nM,  $\pm$ SE) for the controls and 0.8 and 1.5 mg/kg groups were  $5.2 \pm 1.9$ ,  $4.0 \pm 1.7$ , and  $3.5 \pm 1.4$ , respectively. [ $^3\text{H}$ ]GBR 12935  $B_{\text{max}}$  at  $t = 1$  was not significantly different from that of controls at PM doses of 100 and 200 mg/kg (Fig. 2). At  $t = 1$  day,  $K_d$  values (nM,  $\pm$ SE) for the controls and 100 and 200 mg/kg groups were  $6.2 \pm 2.2$ ,  $5.2 \pm 2.7$ , and  $6.2 \pm 2.4$ , respectively. The  $K_d$  of [ $^3\text{H}$ ]GBR 12935 was not significantly different from that of controls for any dose or time groups ( $P > 0.05$ , ANOVA).

Western blotting of DAT protein largely reflected the above [ $^3\text{H}$ ]GBR 12935-binding results for each time period tested. At  $t = 1$ , 14, and 28 days posttreatment, DAT protein was significantly elevated to 115, 140, and 133% of controls, respectively, in the 0.8 mg/kg treatment group (Fig. 3).

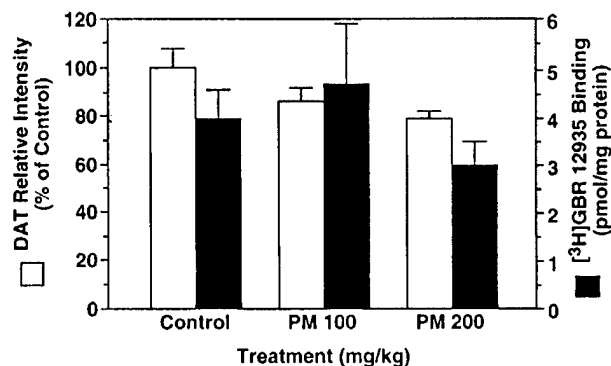


Fig. 2. DAT Western blot densitometry and [<sup>3</sup>H]GBR 12935 binding in C57B1/6 mice sacrificed 1 day after the final treatment with 100 or 200 mg/kg PM. No statistically significant differences were observed (*t* test, *P* > 0.05).

The 1.5 mg/kg treatment group did not show a significant induction of DAT protein until the *t* = 28 time period, where it was enhanced to 145% of control (Fig. 3). A similar effect on DAT was observed in mice treated with 200 mg/kg PM (Fig. 4). As before, DAT protein was not significantly different at 24 h posttreatment; however at *t* = 14 and *t* = 28, DAT protein was significantly upregulated to 135 and 147% of control, respectively, by PM (Fig. 4).

In an effort to determine the threshold dose of PM for up-regulating DAT protein expression, doses of 0.1, 0.2, or 0.4 mg/kg PM were administered according to the standard dosing scheme and sacrificed at *t* = 1, 14, or 28 days posttreatment. At *t* = 1, there was a significant increase in DAT protein at doses of 0.2 and 0.4 mg/kg, but not at 0.1 mg/kg. This effect on DAT protein was persistent for both doses at both 2 and 4 weeks. Again, in the highest dose group, there was a significant increase in DAT protein from *t* = 1 to *t* = 14, which then remained constant until *t* = 28 days. Thus, the threshold dose for significant up-regulation of DAT protein by technical PM was 0.2 mg/kg (Fig. 5) under the dosing regime used in this study.

$\alpha$ -Synuclein protein was also quantified by Western blot-

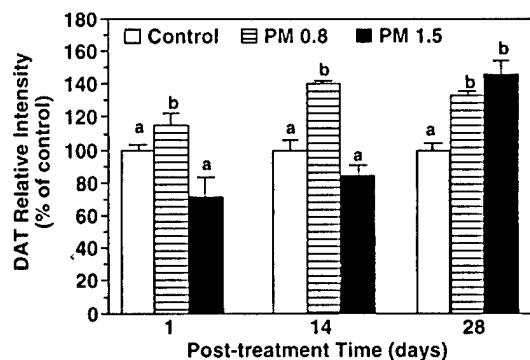


Fig. 3. Time course experiments of DAT Western blot densitometry in C57B1/6 mice treated with 0.8 or 1.5 mg/kg PM. ANOVA was performed on the raw data before calculation as percentage of control. ANOVA and means separation as described for Fig. 1.

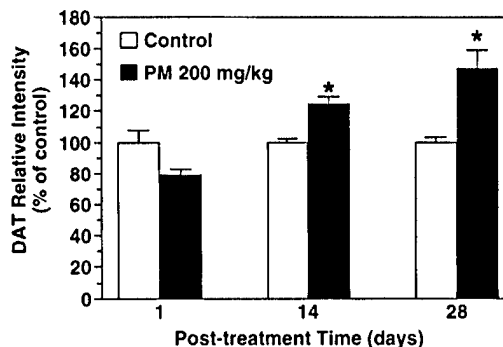


Fig. 4. Time course studies using densitometry of DAT Western blots of striatal synaptosomes from mice treated with 200 mg/kg PM. Raw data were analyzed by *t* test before calculation as percentage of control, and bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different (*P* < 0.05).

ting in the same 0.8 and 1.5 mg/kg dose groups used to assay the DAT (Fig. 6). At a PM dose of 1.5 mg/kg,  $\alpha$ -synuclein was up-regulated to 180% of control in the mice sacrificed 1 day after the last PM dose. However, unlike DAT expression, this effect was not persistent, as evidenced by the lack of any increase in either the 14- and 28-day mice (Fig. 6). At *t* = 1,  $\alpha$ -synuclein protein expression demonstrated a bell-shaped curve over a wide range of PM doses from 25 to 200 mg/kg, with a peak increase at 50 mg/kg to 244% of control.  $\alpha$ -Synuclein protein was also significantly up-regulated at doses of 25 and 100 mg/kg PM (145 and 137% of controls, respectively), but was not significantly changed at the 200 mg/kg PM dose (Fig. 7).

An antibody to synaptophysin, a neuronal vesicle-bound protein, was used in Western blotting techniques to verify uniform loading of protein in Western blots. Synaptophysin protein remained unchanged across treatment groups in all tissues tested, as opposed to the up-regulation observed for DAT and  $\alpha$ -synuclein (Fig. 8). Since staining across all DAT bands was increased, all were included in the quantitation. Uniform protein loading was also ensured by semi-quantitatively measuring the Ponceau-S staining of each Western blot lane, which again remained constant across all treatment groups and time points (data not shown).

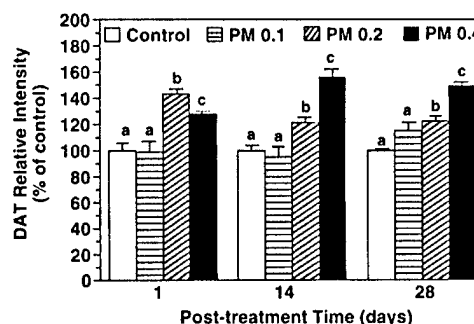


Fig. 5. Time course studies using densitometry of striatal synaptosomes on DAT Western blots from mice treated with either 0.1, 0.2, or 0.4 mg/kg PM. ANOVA and means separation as described for Fig. 3.

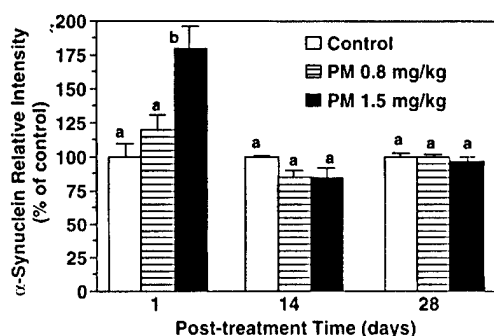


Fig. 6. Densitometry of  $\alpha$ -synuclein in Western blots of striatal synaptosomes from mice treated with either 0.8 or 1.5 mg/kg PM at 1, 14, and 28 days posttreatment. ANOVA and means separation as described for Fig. 3.

## Discussion

The present study confirms and extends the results of Karen et al. (2001), and establishes a potent, slowly developing up-regulation of the DAT by the pyrethroid, PM. In general, pyrethroids are quickly metabolized and excreted from the body, although some pyrethroid metabolites may be persistent (Casida et al., 1983). In light of this rapid metabolism, it was surprising to us that at doses as low as 0.2 mg/kg PM, DAT protein was significantly induced at 4 weeks posttreatment. Thus, DAT expression can occur at doses about an order of magnitude below those previously reported to have this effect (Karen et al., 2001). The initial doses of PM selected for this study, 0.8 and 1.5 mg/kg given 3 times over 2 weeks, are consistent with a reported dermal dose of 0.13 mg/kg/day to humans exposed during the first Gulf War (Abou-Donia et al., 2001). It is also noteworthy that of the four PM stereoisomers, only one of them (1*R*-*cis* configuration across the cyclopropane ring) has significant acute toxicity to mammals (Casida et al., 1983). If this isomer is also solely responsible for the observed effects on

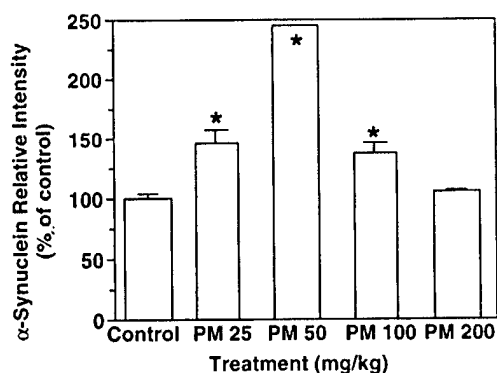


Fig. 7. Densitometry of  $\alpha$ -synuclein in Western blots of striatal synaptosomes from mice treated with increasing doses of PM. Letters denote results of a *t* test, where bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different from controls ( $P < 0.05$ ). The SEM of the 50 mg/kg group was too small to be seen at the y-axis scaling of the graph.

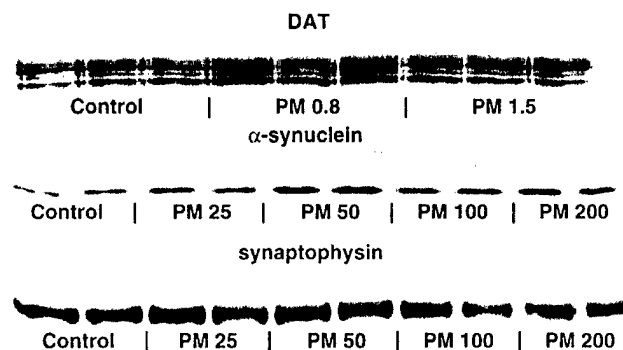


Fig. 8. Examples of representative Western blots of DAT,  $\alpha$ -synuclein, and synaptophysin. Treatments are labeled below stained bands, and are mg/kg PM.

the DAT, then the actual doses of PM responsible for this effect are 4-fold lower than those used in this study. Support for this conclusion is found in published studies with the related pyrethroid, deltamethrin. This compound is composed of a single 1*R*,*cis*, $\alpha$ *S* isomer that is toxic to mammals (murine ip LD<sub>50</sub> = 10 mg/kg, Casida et al., 1983) and it up-regulates DA transport in ex vivo synaptosomes (Kirby et al., 1999).

It is compelling that our published DA uptake data, in which transport is increased at low PM doses (Karen et al., 2001), are mirrored by the [<sup>3</sup>H]GBR 12935-binding studies and quantitation of DAT protein by Western blotting of this study. However, there has been some debate over the use of [<sup>3</sup>H]GBR 12935 as a DAT-specific ligand. Pristupa et al. (1994) found that binding of [<sup>3</sup>H]GBR 12935 in human COS-7 cells transfected with human DAT was not saturable up to 22 nM. It has also been shown that [<sup>3</sup>H]GBR 12935 is able to bind to dopamine-insensitive "piperazine acceptor sites," one of which has been identified as human CYP2D6 (Allard et al., 1994; Hiroi et al., 1997). In our experiments, [<sup>3</sup>H]GBR 12935 binding was specific and saturable, showed excellent correlation with other biomarkers as noted above, and yielded a  $B_{\max}$  value for C57B1/6 control mice of about 3 pmol/mg protein, similar to values reported by Horn (1990) for rat striatum (5.5 pmol/mg) or human caudate tissue (2.7 pmol/mg).

The lack of a statistically significant response at the 1.5 mg/kg dose until  $t = 28$  in the binding and Western blot studies was surprising, since the maximal increase in DA uptake in other cohorts of mice occurred at this dose 1 day posttreatment. It is likely that there are cohort-related differences in the effectiveness of the 1.5 mg/kg PM dose. This possibility is supported by our previous data in which up-regulation of DAT protein did occur at the 1.5 mg/kg dose at 1 day posttreatment (Bloomquist et al., 2002). A slowly developing effect on DAT was observed across all doses tested, since DAT protein was induced to a greater extent in the 14- and 28-day groups vs the 1-day posttreatment groups given 0.2–0.4 mg/kg (Fig. 5) and 200 mg/kg PM (Fig. 4). Thus, induction of DAT up-regulation is variable or

delayed until 28 days posttreatment, when it apparently stabilizes. At high doses of PM (e.g., 200 mg/kg), the delayed response in DAT up-regulation, as well as reduced DA uptake, may be due to nerve terminal toxicity, since this dose causes a reduction in mitochondrial function in previous studies (Karen et al., 2001). After a recovery period, DAT up-regulation again becomes evident. Persistent DAT up-regulation was also noted in Sprague-Dawley rats exposed to heptachlor in the gestational, perinatal, and adolescent stages of development. In this case, DAT binding to [<sup>3</sup>H]mazindol remained significantly increased into adulthood (Purkerson-Parker et al., 2001).

Unlike DAT, the up-regulation of  $\alpha$ -synuclein protein was not a persistent effect, and it had returned to normal levels in the 14- and 28-day treatment groups. Mutations in  $\alpha$ -synuclein are believed to cause the fibrillar aggregates that are the major components of Lewy bodies, a pathological hallmark of PD (Murray et al., 2001). Mutations in the  $\alpha$ -synuclein gene have also been associated with some forms of familial PD (Langston et al., 1998; Mizuno et al., 2001). Manning-Bog et al. (2001) found the herbicide paraquat (10 mg/kg) to cause up-regulation and increased aggregation of  $\alpha$ -synuclein in C57B1/6 mice. This up-regulation was also not persistent, with protein levels returning to control values by Posttreatment Day 7.

It has been reported that human  $\alpha$ -synuclein can complex with human DAT, causing acceleration of cellular DA uptake and DA-induced cellular apoptosis (Lee et al., 2001). DA itself can be metabolized to toxic free radical species that have neurotoxic effects, including inhibition of mitochondrial respiration (Ben-Shachar et al., 1995). Thus, the combined increase in DAT and  $\alpha$ -synuclein by pesticides such as PM may exacerbate this potential for cellular toxicity, especially if sufficiently prolonged. Our observed up-regulation of  $\alpha$ -synuclein is likely a compensatory mechanism, since it has been reported that  $\alpha$ -synuclein is able to inactivate c-Jun N-terminal kinase (JNK), thus protecting cells against oxidative stress (Hashimoto et al., 2002). Although the signaling pathway for protein up-regulation by pyrethroids is unknown, these compounds have a high-affinity interaction with the  $\beta$ -subunit of GTP-binding proteins (Rossignol, 1991a, 1991b), and are reported to increase protein phosphorylation levels by enhancing the effects of both protein kinase C (Enan and Matsumura, 1992) and protein kinase A (Matsumura et al., 1989). The bell-shaped dose-response relationships observed for  $\alpha$ -synuclein suggest that the signaling pathways are modified at different threshold doses, and that the pathway(s) apparently shuts down at higher doses, at least at  $t = 1$ .

To date, studies of pyrethroid effects on dopaminergic pathways have emphasized doses that result in acute intoxication. Electroencephalographic recordings found that pyrethroid-induced intoxication in the rat seemed to originate in neurons of the caudate nucleus and the dopaminergic neurons of the substantia nigra before spreading to other brain regions (Ray and Cremer, 1979; Ray, 1980). Thus,

acutely toxic doses of pyrethroids affect regions of the brain involved in PD, and because they are the first affected, these areas would seem to display an exceptional sensitivity to the action of pyrethroids. Acute treatments of pyrethroids at high doses caused a slight decrease in striatal DA levels (Husain et al., 1991), but a significant increase of 23–37% in levels of DOPAC (Doherty et al., 1988), suggesting an increase in DA turnover. At 200 mg/kg, PM had no effect on striatal DA or DOPAC levels in the treatment paradigm we used (Karen et al., 2001). The low dose effects we show here are more likely to mirror body burdens found in humans, and it remains to be determined whether sustained exposures to low doses can result in loss of striatal DA content, a cardinal sign of PD (Hornykiewicz and Kish, 1986).

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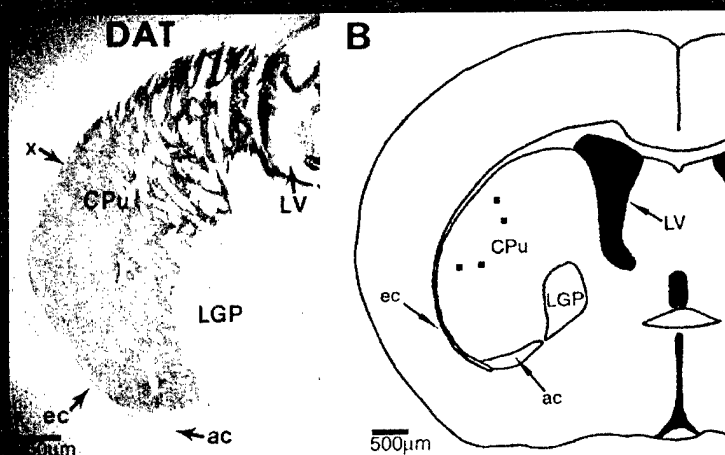
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# Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin

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Epidemiological studies have linked insecticide exposure and Parkinson's disease. In addition, some insecticides produce damage or physiological disruption within the dopaminergic nigrostriatal pathway of non-humans. This study employed immunohistochemical analysis in striatum of the C57BL/6 mouse to clarify tissue changes suggested by previous pharmacological studies of the pyrethroid insecticide permethrin. Dopamine transporter, tyrosine hydroxylase, and glial fibrillary acidic protein immunoreactivities were examined in caudate-putamen to distinguish changes in amount of dopamine transporter immunoreactive protein from degeneration or other damage to dopaminergic neuropil. Weight-matched pairs of pesticide-treated and vehicle-control mice were dosed and sacrificed on the same days. Permethrin at 0.8, 1.5 and 3.0 mg/kg were the low doses and at 200 mg/kg the high dose. Brains from matched pairs of mice were processed on the same slides using the avidin-biotin technique. Four fields were morphometrically located in each of the serial sections of caudate-putamen, digitally photographed, and immunopositive image pixels were counted and compared between members of matched pairs of permethrin-treated and vehicle-control mice. For low doses, only 3.0 mg/kg produced a significant decrease in dopamine transporter immunostaining. The high dose of permethrin did not produce a significant change in dopamine transporter or tyrosine hydroxylase immunostaining, but resulted in a significant increase in glial fibrillary acidic protein immunostaining. These data suggest that a low dose of permethrin can reduce the amount of dopamine transporter immunoreactive protein in the caudate-putamen. They also suggest that previously reported reductions in dopamine uptake of striatal synaptosomes of high-dose mice may be due to nondegenerative tissue damage within this region as opposed to reductions of dopamine transporter protein or death of nigrostriatal terminals. These data provide further evidence that insecticides can affect the primary neurodegenerative substrate of Parkinson's disease.

**Keywords** Dopamine Transporter, Glial Fibrillary Acidic Protein, Parkinson's Disease, Permethrin, Striatum, Tyrosine Hydroxylase

Despite a wealth of investigation into Parkinson's disease (PD), the etiology of the disorder remains elusive. Although there is agreement on a significant genetic contribution in some forms of early onset Parkinson's-like disorders, the role of genetic inheritance in the most common form of the disease, adult- or late-onset PD, remains equivocal (Olanow and Tatton 1999; Tanner et al. 1999; Sveinbjornsdottir et al. 2000; Mouradian 2002). This, in addition to the fact that the xenobiotic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used to produce one of the principal mammalian models for Parkinson's disease (Royland and Langston 1998; Schmidt and Ferger 2001), has prompted a great deal of work on potential environmental causes or triggers for PD.

Insecticides are a widely used class of environmental chemicals that induce their toxic effects by acting on the nervous system. A number of epidemiological studies support a link between insecticide exposure and Parkinson's disease in humans (Semchuck, Love, and Lee 1992; Butterfield et al. 1993; Gorell et al. 1998; Corrigan et al. 2000; Zorzon et al. 2002). Furthermore, a number of recent experimental studies have shown that some insecticides can produce damage or physiological disruption within the dopaminergic nigrostriatal pathway (Bloomquist et al. 1999; Kirby, Castagnoli, and Bloomquist 1999; Miller et al. 1999; Betarbet et al. 2000; Karen et al. 2001; Purkerson-Parker, McDaniel, and Moser 2001; Kirby, Barlow, and Bloomquist 2001). This pathway is the principal focus of degeneration in idiopathic PD (DiMonte and Langston 1995; Poewe and Wenning 1998).

The synthetic pyrethroid insecticides are derivatives of the natural insecticidal pyrethrins found in the chrysanthemum. The principal target of the pyrethroids, in both insects and mammals, is the voltage-gated sodium channel (Ghiasuddin and Soderlund 1985; Ray 2001). The opened state of the channel is prolonged, which produces a hyperexcitability within the target tissue. There is some evidence that other sites in excitable tissues may be

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affected as well, such as voltage-gated calcium channels (Hagiwara, Irisawa, and Kameyama 1988; Duce et al. 1999),  $\gamma$ -aminobutyric acid receptors (Lawrence, Gee, and Yamamura 1985; Bloomquist, Adams, and Soderlund 1986) and neurotransmitter membrane transporters (Kirby, Castagnoli, and Bloomquist 1999; Karen et al. 2001; Bloomquist et al. 2002).

Pyrethroids have been reported to affect the function of components of the dopaminergic nigrostriatal pathway, sometimes in a selective fashion. For example, in rats, decamethrin produced effects on electroencephalographic recordings in striatum and substantia nigra prior to affecting other brain regions (Ray 1980). In addition, deltamethrin-induced increases in blood flow were greater in caudate nucleus compared with cerebral cortex (Ray 1982). In rabbit brain slices, fenvalerate was capable of inducing the release of dopamine and acetylcholine from striatum, but did not cause release of norepinephrine or acetylcholine from hippocampus (Eels and Dubocovich 1988). In neurotransmitter release studies using preloaded synaptosomes, it has been shown that deltamethrin induces dopamine release from striatal synaptosomes at  $EC_{50}$  values 2.4- to 8.6-fold more potent than cortical synaptosomes containing serotonin or glutamate, respectively (Kirby, Castagnoli, and Bloomquist 1999; Bloomquist et al. 2002). Pyrethroids have also been shown to increase the concentration of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum (Doherty et al. 1988). In prenatally exposed rat pups examined as adults, a fenvalerate-induced increase in DOPAC and DOPAC/dopamine ratio suggested an enhanced activity of the dopaminergic nigrostriatal pathway that was not seen for noradrenergic or serotonergic systems (Lazarini et al. 2001). The fairly limited number of studies on any given pyrethroid, in addition to the variety of dependent variables employed, suggest it would be premature to consider a specific pyrethroid as a reliable, positive experimental control for pyrethroid-induced insult to the nigrostriatal pathway. Nevertheless, there appears to be sufficient evidence to warrant further exploration of the role of pyrethroids in such damage.

Pyrethroid insecticides have also been shown to modulate neurotransmitter uptake (Kirby, Castagnoli, and Bloomquist 1999; Karen et al. 2001; Bloomquist et al. 2002). A recent study has demonstrated that the type I pyrethroid permethrin can alter maximal dopamine uptake ( $V_{max}$ ) by mouse striatal synaptosomes (Karen et al. 2001). At low doses  $V_{max}$  was increased, whereas at higher doses  $V_{max}$  was decreased, as was open field behavior, compared to vehicle controls. The lack of an accompanying change in  $K_M$  suggested that these pharmacological changes reflected an alteration in the amount of dopamine transporter (DAT) present within the synaptosomal tissue sample, rather than a change in the efficiency of the transporter. Furthermore, the reversal of increased maximal dopamine uptake seen at the high doses of permethrin could reflect damage or degeneration of dopaminergic neuropil within the striatum. In the present study, immunohistochemical techniques were employed in the mouse striatum to clarify the tissue changes suggested by the aforementioned kinetic studies. This technique permitted the

evaluation of changes in a topographically defined region of the striatum: that which contains the highest density of nigrostriatal dopaminergic afferents (Heimer, Zahm, and Alheid 1995) and the region most likely to be affected in PD (Kish, Shannak, and Hornykiewicz 1988; Graybiel, Hirsch, and Agid 1990; Miller et al. 1999). DAT, tyrosine hydroxylase (TH), and glial fibrillary acidic protein (GFAP) immunoreactivities were examined in an attempt to distinguish changes in the amount of the DAT from degeneration or other damage to dopaminergic striatal neuropil.

## MATERIALS AND METHODS

### Animals and Treatments

Male C57BL/6 retired breeder mice were used because this study attempted to assess the effects of permethrin on the nigrostriatal pathway and the C57BL/6 mouse is the most common, and sensitive, rodent model used in studies of chemically induced parkinsonism (Heikkila, Hess, and Duvoisin 1984; Royland and Langston 1998). Mice were 7 to 9 months old at the time of the experiment and were obtained from Harlan Sprague-Dawley, Dublin, VA, USA. They were assigned to groups by weight, such that every mouse designated for insecticide treatment had a paired, weight-matched control. These weight-matched pairs were randomly assigned to different dosage groups. Insecticide-treated mice were given intraperitoneal (IP) injections of permethrin (Sigma Chemical, St. Louis, MO, USA) in a methoxytriglycol vehicle, whereas control mice received vehicle alone. The permethrin compound was 96.6% pure and was comprised of 71.8% *trans*-isomer and 24.8% *cis*-isomer. Regardless of dose, all mice received three injections over a 2-week period according to the methods of Bloomquist et al. (1999) and Karen et al. (2001), with day 1 being the first injection, followed by injections on days 8 and 15 and sacrifice on day 16. For every permethrin-treated mouse that was sacrificed on a given day, its weight-matched, vehicle-control partner was also sacrificed. Mice receiving 0.8, 1.5, or 3.0 mg/kg of permethrin were considered the "low-dose" group and those receiving 200 mg/kg were considered the "high-dose" group. These doses were selected because permethrin treatments of similar magnitude were previously shown to produce the greatest changes in dopamine uptake in the mouse striatal synaptosome preparation (see Karen et al. 2001). In addition, the extent of the dosing spectrum was limited to these values in order to maximize resources and minimize the number of mice used in the experiment.

### Fixation, Tissue Sectioning, and Histochemistry

On the day of sacrifice, the permethrin-treated and matched vehicle-control mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with a phosphate-buffered saline (PBS) rinse (0.05 M, pH 7.4), followed by 4% paraformaldehyde fixative solutions (in 0.1 M phosphate buffer) at respective pH values of 7.0 and 10.5. Brains were then removed and post-fixed in the pH 10.5 fixative for 4 hours, rinsed three

times with PBS, and cryoprotected overnight in 10% sucrose at 4°C.

Using a cryostat, 16- $\mu$ m sections through the striatum were cut onto slides beginning at the rostral-most appearance of the lateral ventricle. For the high-dose mice, three consecutive sections were cut to provide exposure to TH antibody (Protos Biotech, New York, NY, USA, formerly Eugene Tech, catalogue no. CA-101), cresyl violet staining, and either DAT antibody or GFAP antibody (DAKO, Glostrup, Denmark, catalogue no. Z0334). For the low-dose mice, two consecutive 16- $\mu$ m sections were cut, respectively, for exposure to DAT antibody (Chemicon International, Temecula, CA, USA, catalogue no. AB1591P) or cresyl violet staining. Sections for TH and GFAP staining were not prepared for this condition because previous work provided no reason to suspect death or damage of striatal neuropil following the low dose regimen (see Karen et al. 2001). The sectioning sequence was repeated at 320- $\mu$ m intervals, through the striatum, until 12 sets of multiple sections were cut for primary antibody staining. During cutting of the fifth set of slides, a supplemental slide set was prepared (set 5A) from tissue immediately adjacent (within 16  $\mu$ m) to the sections cut for antibody staining. This supplemental set was used as an omission control for primary antibody staining.

Given the operational definition of the cutting procedure described above, sectioning began at approximately 1.94 mm rostral to bregma and ended at approximately 1.68 mm caudal to bregma. The caudate-putamen extends from 1.94 mm rostral to bregma through 2.30 mm caudal to bregma (Franklin and Paxinos 1997). After completing the sectioning of a given permethrin-treated brain, corresponding sections from the matched-vehicle brain were placed on the same slide, which insured identical histochemical treatment for both sections. The two sectioned brains comprised a single case. The slide positions of the treated and vehicle-control brains were counterbalanced across cases, as well as the order in which the brains were cut.

Because the two brains were cut in series, as opposed to parallel within the same block, it was possible that there were minor variations in section thickness that could have affected the subsequent quantification of stained neuropil described below. However, the counterbalancing of the order in which brains were cut would help to distribute such error randomly. Furthermore, variation in section thickness is likely to occur across serial sections within a given brain and not just across different brains, further ensuring a random distribution of this variability.

Immunohistochemistry was performed directly on the slides according to a modification of methods previously outlined by this laboratory (Klein and Blaker 1990; Klein et al. 1992; Misra and Klein 1995). Each antiserum used in this study was commercially available and has been used in a multitude of studies on the nervous system, many of which involve analysis of striatal tissue (e.g., Lew et al. 1992; Pu et al. 1994; Freed et al. 1995; Kordower et al. 1996; Vaughan et al. 1996; Wirth, Rufer, and Unsicker 1996; Betarbet et al. 1997; Zimmer, Ennis, and Shipley 1997; Sortwell, Collier, and Sladek 1998; Sugama et al. 2003). Specifi-

cificity of these antibodies has been previously verified by others using a variety of techniques including Western blot, enzyme-linked immunosorbent assay (ELISA), immunoelectrophoresis, and immunohistochemistry. Briefly, sections were rinsed with PBS, incubated with 3% hydrogen peroxide to quench endogenous peroxidase, and rinsed again. The slides were then incubated sequentially in (1) 10% normal goat blocking serum containing 0.15% Trinton X-100 and (2) polyclonal rabbit antisera to either DAT (1:600), TH (1:400), or GFAP (1:6400), overnight, at room temperature. Tissue was then processed by the avidin-biotin complex method using a Vectastain Elite kit (Vector Labs, Burlingame, CA, USA, catalogue no. PK6101) and 0.05% diaminobenzidine in 0.01% hydrogen peroxide as the chromogen. Sections were then dehydrated in alcohols, cleared in xylene, and coverslipped. Alternate sections were stained with 0.3% cresyl violet in 50% ethanol. Diluent for primary and secondary (biotinylated goat anti-rabbit, included in the kit) antibody solutions was 1% goat serum in PBS containing 0.15% Trinton X-100. Tissue incubations were performed in a humidity chamber. Immunostaining produced with the three primary antibodies can be seen in Figures 1 and 2. These photographs are simply intended to show examples of the general quality of tissue staining obtained in the study. However, they also serve as evidence of positive and/or negative controls for primary antibody staining. The region of the caudate-putamen, a region known to be rich in dopaminergic afferents, exhibits dense immunoreactivity for both the DAT and TH antibodies in Figures 1 and 2. Furthermore, there is a drastic reduction of this staining at the medial and lateral borders of the caudate-putamen, in the respective regions of the lateral globus pallidus and the external capsule. This can be seen for DAT in Figures 1 and 2, and the reduction in TH at the external capsule can be seen in Figure 2. This lateral portion of the globus pallidus and the external capsule are regions where dopaminergic input is known to be limited (Parent and Smith 1987). Immunopositive staining in the above-noted primary antibody omission-control sections was virtually absent.

#### Morphometric Location of Fields for Image Analysis

An individual data point in this study was defined as the difference in the amount of immunoreactive neuropil (DAT, TH, or GFAP) between a brain section from a permethrin-treated mouse and the corresponding section from its matched vehicle control, located on the same slide. The amount of immunoreactive neuropil in a given brain section was a mean, calculated from four 3070- $\mu$ m<sup>2</sup> fields, distributed within the dorsolateral portion of the striatum (Figure 1B). This region is the primary striatal target of dopaminergic neurons originating from the rodent substantia nigra pars compacta (Heimer, Zahm, and Alheid 1995). In order to insure consistent sampling between sections from treated and matched vehicle-control sections, a morphometric procedure was used to define the location of the four fields within each brain section. Consistent identification of the caudate-putamen, across sections, was aided by the fact that (1) throughout most of its

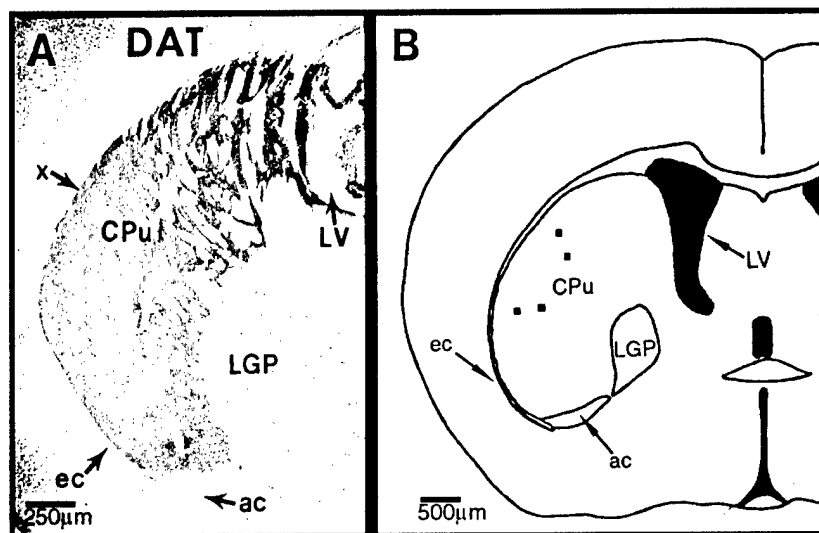


FIGURE 1

(A) Low-magnification image of a coronal section through the striatum showing immunostaining for DAT. The region indicated by the "x" is shown at higher magnification in Figure 2. (B) Schematic diagram of a similar section, modified from a mouse brain atlas (Franklin and Paxinos 1997), showing the location and size ( $3070 \mu\text{m}^2$ ) of each of the four analysis fields in the dorsal lateral portion of the striatum. Both images also illustrate prominent anatomical landmarks in the region. CPu = caudate-putamen; ec = external capsule; LV = lateral ventricle; ac = anterior commissure; LGP = lateral globus pallidus.

rostrocaudal course, the caudate-putamen is bordered dorsally, ventrally, and laterally by the external capsule, a well-defined band of white matter; (2) the dorsomedial border of the nucleus is adjacent to and can be easily distinguished from the cavity of the lateral ventricle; and (3) the ventromedial border of the nucleus can be histologically distinguished from the less densely staining globus pallidus (see Figure 1A) in both immunostained and cresyl violet-stained sections (Franklin and Paxinos 1997). In the rostral third of the nucleus, where the external capsule does not extend to the ventral border, the lateral striatal stripe and the anterior commissure were used to locate the ventral border of the caudate-putamen and distinguish it from the nucleus accumbens.

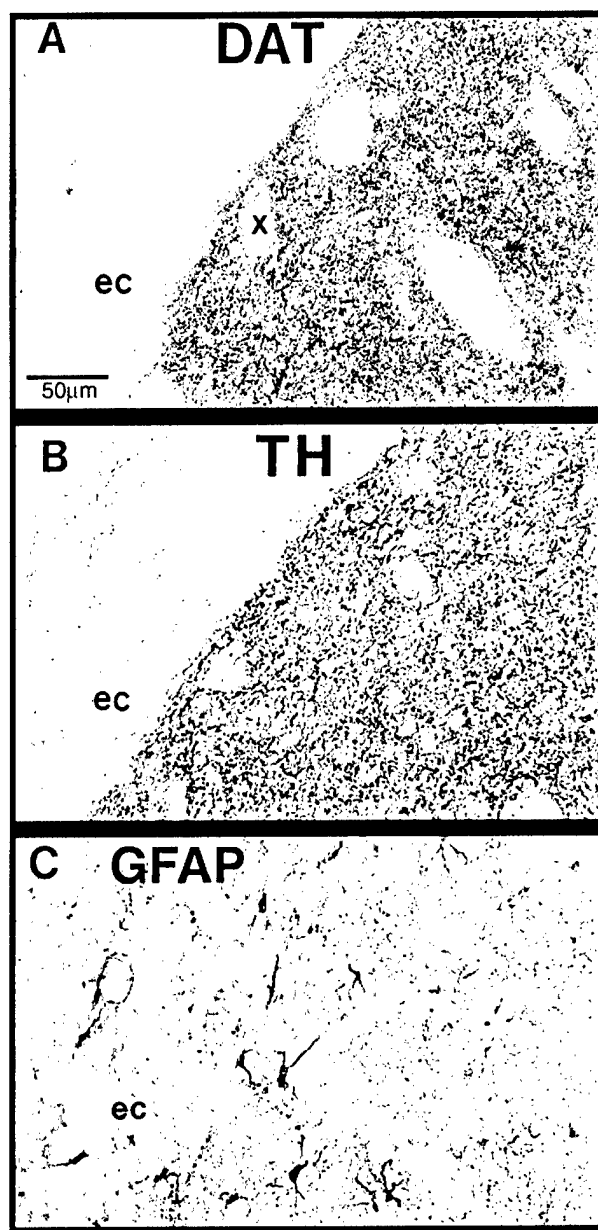
Morphometric location of the four analysis fields was done on camera-lucida tracings of brain sections. Initially, a central point of the caudate-putamen was operationally defined as follows: The dorsal and ventral tips of the nucleus were located with the aid of the external capsule. At the dorsoventral midpoint of the nucleus, a horizontal line was drawn extending from the external capsule laterally, to the pallidal border medially. The midpoint of this line was operationally defined as the center point of the caudate putamen.

After operationally locating the center point of the caudate-putamen, the outer circumference of the nucleus was traced from the ventral-most tip to the dorsal border with the lateral ventricle. Along this arc, marks were made at  $1/3$  and  $2/3$  of the total distance. Lines were then drawn from these marks to the center

point of the nucleus. Along each of these two radii, marks were made at  $1/4$  and  $1/2$  the distance between the perimeter of the nucleus and the center point. This yielded four marks, designating the centers of the four fields that were to be digitally photographed for image analysis (Figure 1B). Around these fields, numerous tissue landmarks were traced, such as unstained fiber bundles or imperfections, to aid in relocating these fields for photography. The fields identified by the procedure described above were consistently distributed within the dorsolateral quadrant of the caudate-putamen and permitted measurements from similar regions within permethrin-treated and matched vehicle-control brains.

As alluded to above, and as can be seen in Figures 1 and 2, coronal sections through the striatum are characterized by fairly numerous, unstained fiber bundles of passage, the pattern of which varies among individual animals. In order to minimize the effect of these fibers of passage upon the analysis, when such a bundle fell within the measurement field designated for photography, the position of the field was adjusted to incorporate the closest adjacent field of homogenous immunolabeling. Thus, the fields such as those depicted in Figure 1 could vary by as much as  $50 \mu\text{m}$  in any direction, but all still remained within the dorsolateral quadrant of the caudate-putamen.

After morphometrically locating and photographing the four analysis fields from the caudate-putamen, the convergence of the mediolateral and dorsoventral midpoints of the corpus callosum was visually located and photographed in each brain section.



**FIGURE 2**

(A) Higher-magnification image of a portion of the section shown in Figure 1A. The "x" in both figures indicates the same tissue location. (B and C) Similar locations within the caudate-putamen, relative to A, showing staining for TH and GFAP, the other two antibodies used in the study. The anatomical landmark of the external capsule is indicated in all three photomicrographs. DAT = dopamine transporter; TH = tyrosine hydroxylase; GFAP = glial fibrillary acid protein; ec = external capsule.

This allowed assessment of a possible change in GFAP immunolabeling in a nonstriatal region and addressed whether changes in GFAP immunoreactivity were a generalized response to insecticide exposure. The mediolateral midpoint was located by following an imagined, vertically oriented line, continuing from the dorsal median fissure (the fissure that separates the two cerebral hemispheres) through the corpus callosum. At this midline location, the dorsal and ventral borders of the callosum could be easily recognized and the midpoint between these borders was visually located. A digital photograph was then taken that was centered on this point.

#### Image Analysis

The amount of immunostained neuropil in photographs of each of the four fields within a section was quantified using the "Histogram" feature of Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA, USA). Digital images of a given size are comprised of a fixed array of loci called pixels. For any selected area of pixels from a digital photograph, the "Histogram" function can determine the mean grayscale value (0 to 255, where 0 is black and 255 is white) and the number of pixels that are darker than a specified grayscale value.

A threshold value for immunostained neuropil was determined by sampling the mean grayscale value of varicosities, which were visually judged to be unambiguously immunopositive, within every brain section of the vehicle-control mice. The grayscale value for two such varicosities was averaged for each field and the mean across the four fields was taken as the average value for the section. The mean across all sections from each vehicle-control mouse was determined, and the mean grayscale value across all vehicle-control mice was used as the threshold value for identifying immunostained neuropil in both vehicle-control mice and permethrin-treated mice. Image pixels as dark, or darker than this value, were then considered as immunopositive labeling, irrespective of how much darker they were. Pixels lighter than this threshold value were not counted. Using the above-noted threshold value, the number of pixels darker than threshold was counted for each photograph of the four measurement fields from each striatal section. This value was averaged across the four measurement fields from each section and was considered the amount of immunostained neuropil in that section. These immunopositive pixel counts were then compared between vehicle and insecticide-treated mice. The determination of immunopositive labeling and statistical analysis for the callosal field was performed in the same fashion.

The typical distribution of grayscale values for the pixels from a photograph of a given measurement field was bell-shaped. A random sample of 50 photographs from among all vehicle-treated mice in the low-dose condition revealed that, on average, the darkest 22% of DAT-stained pixels were being counted. Although this probably reduced the chance of detecting real differences between matched vehicle- and insecticide-treated mice, it

likely minimized the probability that any differences were attributable to variation in nonspecific background staining, even though such staining appeared minimal in omission controls.

### Statistical Analysis

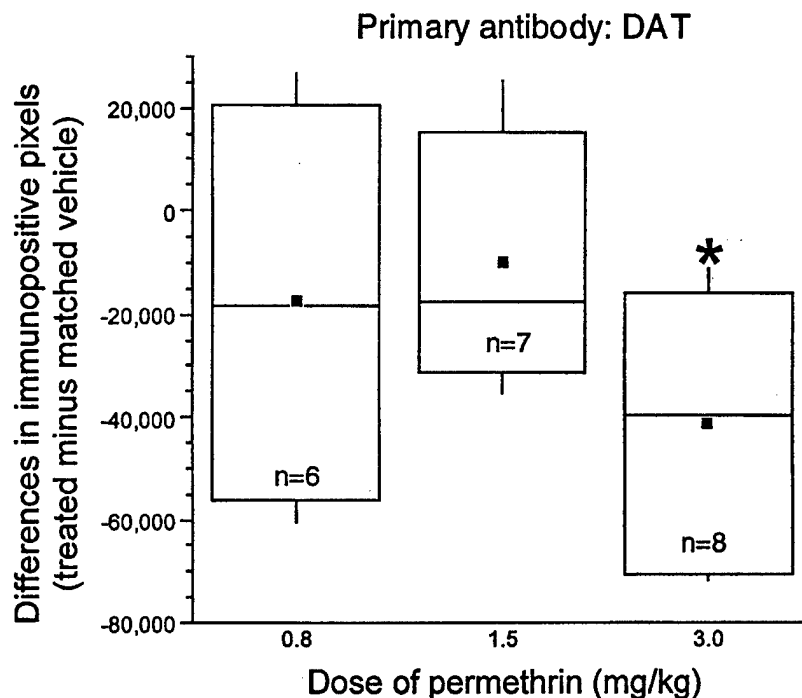
As alluded to above, on each microscope slide, a brain section from a permethrin-treated animal was paired with a section from a matched vehicle-control animal. These mice were injected on the same days, sacrificed on the same day, and the sections were taken from similar rostrocaudal positions within the striatum. In addition, the paired sections on a slide shared identical tissue processing conditions. Therefore, the individual data points subjected to statistical analysis in this study were the differences in immunopositive staining (pixel counts), as described above, between the paired permethrin-treated and matched vehicle-control sections on a microscope slide.

Initially, scatterplots were used to assess the effects of microscope slide section position and date of tissue processing on differences in immunostained neuropil between paired sections. Based on this analysis, an analysis of variance (ANOVA) model, fitted using the GLM procedure of SAS (SAS Institute,

Cary, NC, USA), was used to correct for the effect of processing date on each data point. The corrected differences in immunostaining were then consistent with the assumptions for analysis by a paired *t* test. For a pair of permethrin-treated and matched vehicle-control brains, the difference in immunostained neuropil was averaged across microscope slides. Then, for each dose concentration group, the grand mean of these corrected mean differences was tested for its difference from zero using an alpha level of 0.05. Inflation of the type I error rate was avoided by treating each dose concentration group as a separate experiment.

### RESULTS

Figure 3 is a box and whisker plot of the distribution of mean differences in DAT immunostaining, between matched pairs of permethrin-treated and vehicle-control mice, within each of the "low-dose" concentration groups. The tips of the whiskers respectively represent the minimum and maximum mean differences, whereas the length of the box represents the interquartile range. The line and solid square within each box respectively represent the median and grand mean of the distribution



**FIGURE 3**

Distributions of differences in immunopositive staining for DAT, between pairs of pesticide-treated and matched vehicle-control mice, for each of the low doses of permethrin. For each box and whisker plot, whisker tips respectively represent minimum and maximum differences whereas box length shows the interquartile range of the differences. The line and black square respectively indicate the median and mean of the difference scores. The asterisk indicates a mean of difference scores significantly different from zero. *n* = number of matched pairs of permethrin-treated and vehicle-control mice.

of mean differences. The number of matched pairs of treated and vehicle-control mice is also indicated for each dose. A grand mean of zero represents no change in immunostaining between matched pairs of mice. As can be seen in Figure 3, only the 3.0-mg/kg dose of permethrin produced a significant decrease in DAT immunostaining within the caudate-putamen, compared with matched vehicle-control mice ( $df = 7$ ,  $p = .007$ ), although all "low-dose" permethrin-treated mice showed a trend toward decreased labeling. The mean decrease of 41,713 pixels in the 3.0-mg/kg group was 48.6% of the mean number of pixels in the matched vehicle control group. An example of the change in DAT immunopositive staining, that reflects this mean decrease, can be seen in Figures 5C and 5D. Although this percentage provides some idea of the magnitude of change, the reader should note that it is based upon a corrected mean across vehicle-control mice that was not used in the formal statistical analysis of the results. As noted in the Materials and Methods, that analysis was based on *differences* between individual brain sections from treated mice and their matched-vehicle controls that were processed under the same conditions.

Figure 4 is a box and whisker plot of mean differences in DAT, TH, and GFAP immunostaining, between matched pairs of treated and vehicle-control mice, in the "high-dose" concentration group (200 mg/kg permethrin). As seen in the figure, the "high dose" of permethrin did not produce a significant change in DAT or TH immunostaining within the caudate-putamen, compared with matched-vehicle controls, although there was a trend toward a decrease for both types of labeling. However, as in-

dicated by the asterisk, the "high dose" of permethrin resulted in a significant increase in striatal GFAP immunostaining, compared to matched vehicle controls ( $df = 7$ ,  $p = .048$ ). The mean increase of 4524 pixels was 879% of the mean number of pixels in the matched vehicle control group. Again, the value of this percent change should be interpreted in light of the caveat noted above. An example of the change in GFAP immunopositive staining, which reflects this mean increase, can be seen in Figures 5A and 5B.

Comparison of the callosal field between matched pairs of treated and vehicle-control mice in the "high-dose" concentration group (200 mg/kg of permethrin) revealed a mean difference of -10.4 pixels ( $N = 8$ ; median = 51.3; range = 781.8; interquartile range = 200.3). This change, which represented only a 5.3% decrease relative to the mean number of pixels in the matched vehicle-control group, was not significantly different compared with a mean difference of zero.

## DISCUSSION

The results of this experiment provide further support for the notion that environmental chemicals, in this case insecticides, can produce changes in components of the dopaminergic nigrostriatal pathway; a pathway that is the primary neurodegenerative substrate of Parkinson's disease. Taken together with a previous report on mouse striatal synaptosomes, these data indicate that a low dose (1.5 to 3.0 mg/kg) of the pyrethroid insecticide permethrin cannot only alter the kinetics of dopamine uptake

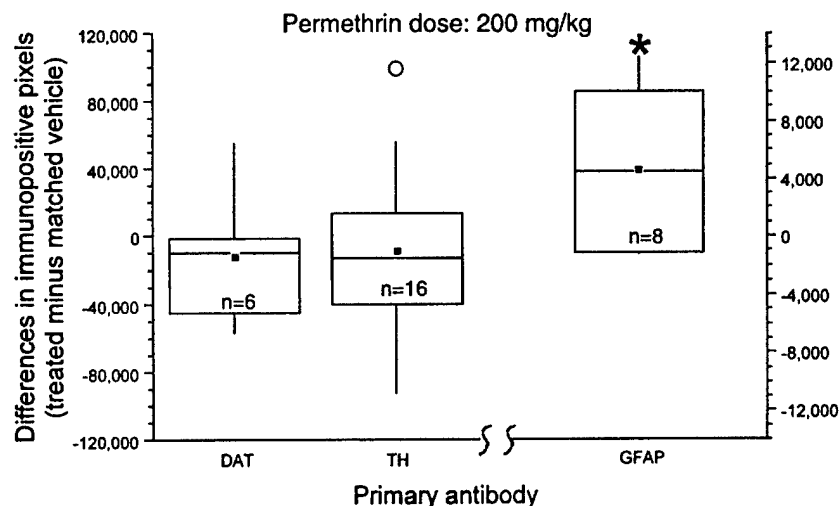


FIGURE 4

Box and whisker plots of differences in immunopositive staining for DAT, TH, and GFAP, between matched pairs of pesticide-treated and vehicle-control mice, for the high dose (200 mg/kg) of permethrin. The *open circle* is a difference score that is more than 1.5 interquartile ranges above the median. The *asterisk* indicates a mean of difference scores significantly different from zero. Note that the y-axis for the GFAP data is drawn to a different scale because the amount of GFAP immunopositive material is normally an order of magnitude more sparse compared with that for DAT and TH.

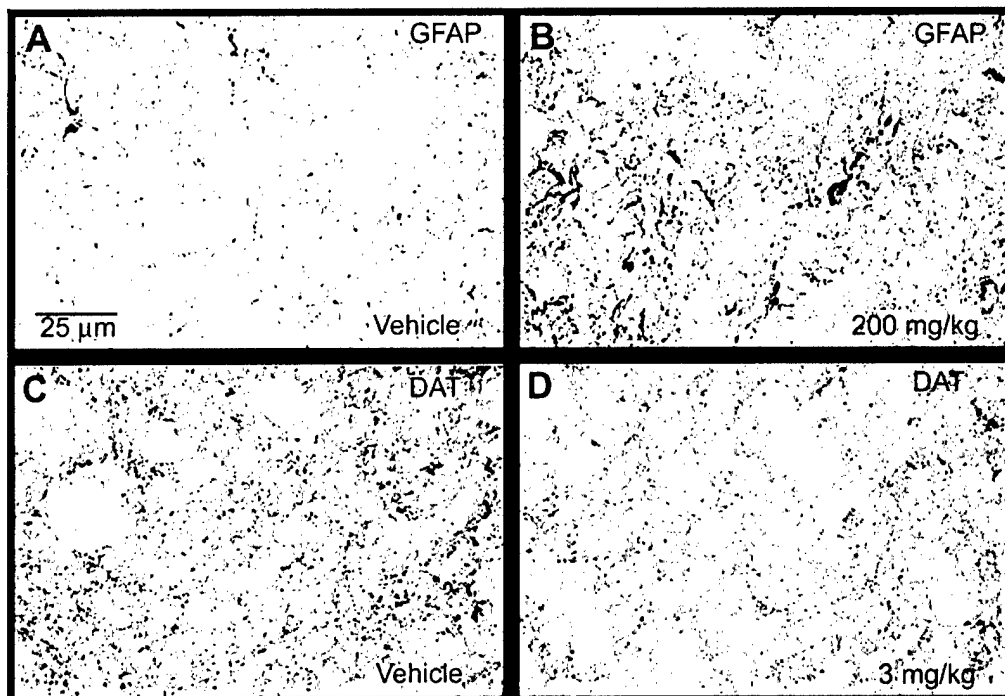


FIGURE 5

High magnification images illustrating the two significant changes in immunoreactivity reported in this experiment. The increase in GFAP immunoreactivity observed at the 200-mg/kg dose of permethrin can be seen by comparing *A* and *B*, whereas the decrease in DAT immunoreactivity is apparent by comparing *C* and *D*. All photographs were made from the dorsolateral sample field, at the same magnification and level of illumination. The quantitative differences in immunoreactivity between the photographs of matched sections from treated and vehicle controls are of the same magnitude as the mean changes in immunoreactivity reported in the text.

(Karen et al. 2001), but can change the amount of DAT immunoreactive protein within the caudate-putamen. Furthermore, high doses of permethrin, which also modify the kinetics of dopamine uptake, can induce glial responses in the caudate-putamen, which have been associated with neuronal tissue damage. The lowest doses at which such changes are observed are more than two orders of magnitude lower than the LD<sub>50</sub> reported for IP administration of the commercial formulation of permethrin known as Ambush (Williamson et al. 1989).

Although low doses of permethrin can affect both dopamine uptake and the amount of DAT immunoreactive protein in components of the dopaminergic nigrostriatal pathway, the effects are in different directions. A 1.5-mg/kg dose of permethrin has been reported to produce an increase in maximal dopamine uptake in striatal synaptosomes (Karen et al. 2001), whereas in the present experiment, 3.0 mg/kg produced a decrease in DAT-immunoreactive neuropil within the caudate-putamen. These two findings would be concordant if an increase in the efficiency of DAT transport overcompensated for a decrease in the amount of DAT protein. However, Karen et al. (2001) failed to find a

change in the  $K_M$  for synaptosomal dopamine uptake, arguing against increased DAT efficiency.

Alternative explanations for the difference in the direction of the two findings noted above relate to differences in the nature of the tissue samples analyzed. For example, in the present immunohistochemical study, anatomical landmarks within transverse forebrain sections were used to select analysis fields within the caudate-putamen, the region of the striatum containing the vast majority of dopaminergic afferents from the substantia nigra (Heimer, Zahm, and Alheid 1995). Furthermore, each of these analysis fields was only on the order of 3070  $\mu\text{m}^2$ . The kinetic data of Karen et al. (2001) was gathered in a synaptosomal preparation made from the entire dissected striatum. This sample may have included varying amounts of tissue from striatal regions outside the caudate-putamen, such as the nucleus accumbens, or nonstriatal regions, such as the globus pallidus, both of which are physically contiguous with the caudate-putamen (Franklin and Paxinos 1997). Therefore, the present study analyzed changes in a smaller sample of tissue, from a more topographically restricted region of the striatum; the



region that is most closely associated with degenerative changes in PD (Kish, Shannak, and Hornykiewicz 1988; Graybiel, Hirsch, and Agid 1990; Miller et al. 1997). This suggests the possibility that different topographic subregions within the striatum may be differentially affected by permethrin exposure. Indeed, it has been shown that portions of the dopaminergic input to the ventral and medial striatum are more resistant to the neurodegenerative effects of MPTP and rotenone compared with the dopaminergic input to the more dorsolateral striatal region containing the caudate-putamen (Gerhardt et al. 1985; Mizukawa, Sora, and Ogawa 1990; Betarbet et al. 2000). In addition, using a whole striatal preparation of mouse synaptosomal membranes, it has recently been reported that low systemic doses of permethrin increased the amount of DAT protein as revealed by Western blot analysis (Bloomquist et al. 2002).

Although the sampling within the present experiment was more restricted with regard to striatal topography, the immunohistochemical analysis most likely surveyed a more extensive portion of individual nigrostriatal neuronal morphology compared with analysis of synaptosomes. Synaptosomal preparations are designed to isolate the synaptic bouton and sometimes contain a portion of the immediate postsynaptic membrane (Webster 2001). Alternatively, any DAT-containing portion of a neuron that lies near the surface of the striatal tissue section is available for labeling by the immunohistochemical procedure. Electron microscopic immunohistochemical studies of the dorsolateral striatum and substantia nigra pars compacta have reported that DAT-immunoreactive protein can be found not only at the plasma membranes of synaptic boutons, but at the membranes of axonal segments lying between the boutons, as well as at somal and dendritic membranes of dopaminergic neurons (Nirenberg et al. 1996; Hersch et al. 1997). Because interbouton axonal segments course through the caudate-putamen, immunohistochemical procedures should label axon-associated DAT, in addition to bouton-associated DAT. Synaptosomal preparations would be expected to primarily sample bouton-associated DAT, because these structures do not contain axonal membrane, and any portion derived from the membrane of a postsynaptic striatal neuron would be almost exclusively non-dopaminergic. Therefore, the regional intraneuronal population of DATs examined in the present study may have differed from that examined in studies using synaptosomal preparations from whole striatum (Karen et al. 2001; Bloomquist et al. 2002). In some dopaminergic neurons, there is a functional differentiation between DATs located in different regions of the cell. For example, dendritic DATs in the substantia nigra are capable of releasing dopamine, through reverse transport, under normal physiological conditions (Falkenburger, Barstow, and Mintz 2001). This potential for functional differentiation between DATs in different parts of the neuron, combined with a probable difference in intraneuronal populations of DATs examined, could also account for the difference in direction of the kinetic data of Karen et al. (2001) and the present immunohistochemical study.

As noted earlier in this article, a decrease in maximal dopamine uptake ( $V_{max}$ ) has been reported for striatal synaptosomes following higher doses of permethrin (25 or 200 mg/kg) (Karen et al. 2001). This suggested that these doses may induce significant death of dopaminergic striatal terminals. The dopamine synthesizing enzyme TH is commonly used as an immunohistochemical marker to identify the presence of dopaminergic neuropil within the striatum (e.g., Gerhardt et al. 1985; Nirenberg et al. 1996, 1997; Ho and Blum 1998; Brooks et al. 1999; Canudas et al. 2000; Betarbet et al. 2000). Although this enzyme is also critical for synthesis of norepinephrine and epinephrine, there is an insignificant presence of neurons that contain these transmitters within the striatum (Aston-Jones, Shipley, and Grzanna 1995; Saper 2000). Therefore, in the "high-dose" portion of the present work, TH immunoreactivity was used as an additional marker for dopaminergic terminal death within the striatum because the digital image analysis, by itself, cannot distinguish between a reduction in DAT-immunoreactive protein alone and the disappearance of DAT-containing terminals within the striatum. It was possible that the DAT protein could be down-regulated without accompanying degeneration of dopaminergic striatal afferents. The lack of change in the amount of DAT- and TH-immunoreactive neuropils, at doses of 200 mg/kg of permethrin, fails to support the notion that dopaminergic terminal death, within the caudate-putamen, is a substrate for the previously reported decrease in synaptosomal  $V_{max}$ . The absence of a decrease in DAT and TH immunoreactivities is consistent with a previous report that this dose did not change striatal levels of dopamine (Karen et al. 2001), which also argues against terminal death being responsible for the decrease in  $V_{max}$ .

Increased GFAP, an intermediate filament protein of astrocytes, has been shown to be a marker of the onset, degree, and locus of neuropathology (Norton et al. 1992; O'Callaghan 1993; O'Callaghan, Jensen, and Miller 1995; Eng, Ghimikar, and Lee 2000). Such increases correspond not only to sites where there is easily identifiable loss of neuropil, such as the striatum following MPTP or 1-methyl-4-phenylpyridinium ( $MPP^+$ ) exposure (Schneider and Denaro 1988; Francis et al. 1995; Canudas et al. 2000; Akari et al. 2001), but where there is histological evidence of tissue damage that is more subtle than frank degeneration (O'Callaghan, Jensen, and Miller 1995). Although the lack of change in TH and DAT immunoreactivities within the caudate-putamen argue against the death of dopaminergic nigrostriatal terminals in the "high-dose" condition, the corresponding increase in GFAP immunoreactivity suggests the possibility of axon-terminal damage that has not advanced to frank degeneration. Such damage may be sufficient to render the DAT inoperative, which would be consistent with the decrease in maximal dopamine uptake reported by Karen et al. (2001). Furthermore, nonfatal damage to the lipid bilayer of striatal synaptic boutons could permit non-transporter-mediated leakage of dopamine from nigrostriatal terminals. This could also account for a decrease in synaptosomal  $V_{max}$ , while leaving DAT and TH

immunoreactivities unchanged. Again, such comparisons with the aforementioned synaptosomal study should be tempered by the possibility of a topographic difference in the striatal tissue samples examined.

It should be noted that the observed increases in GFAP immunoreactivity could also represent damage to glial cells, because such increases have been reported following damage to oligodendrocytes and in astrocytes that survive direct astrocytic insult (Smith, Somera, and Eng 1983; Takada, Li, and Hattori 1990). Although the increase in GFAP immunoreactivity may not be solely attributable to neuronal insult, it does appear to be regionally restricted as opposed to a generalized response to introduction of permethrin into the body. This is suggested by the absence of a significant change in GFAP immunoreactivity within the corpus callosum. Finally, there is evidence suggesting that MPTP-induced damage to the dopaminergic nigrostriatal pathway is capable of inducing an up-regulation of TH protein and mRNA (Greenwood et al. 1991; Bezard et al. 2000; Rothblat, Schroeder, and Schneider 2001), as well as sprouting (Song and Haber 2000), in surviving nigrostriatal neurons, across a variable time frame. If a high dose of permethrin were capable of a similar effect, a reduction in TH immunoreactivity, due to the death of nigrostriatal terminals, could be masked, while still inducing an increase in GFAP. This scenario cannot be ruled out. However, such compensatory effects within the nigrostriatal pathway may be dependent on the type of damage since lesions of this pathway, induced with 6-hydroxydopamine, produced a down-regulation of TH mRNA in surviving nigral neurons (Sherman and Moody 1995).

As noted in Materials and Methods, brain sections were not prepared for TH and GFAP staining in the "low-dose" condition because previous work provided no reason to suspect death or damage of striatal neuropil following the low-dose regimen (see Karen et al. 2001). Therefore, degeneration of dopaminergic terminals within the striatum, and hence, a reduction in their numbers, could not be definitively ruled out as a possible substrate for the decrease in DAT immunoreactive protein observed at the 3.0-mg/kg dose of permethrin. As noted earlier, a decrease in DAT was not seen at the next higher dose of permethrin used in this experiment (200 mg/kg). In light of this, it should be noted that analyses of dopamine uptake into striatal synaptosomes have revealed significant changes at a very restricted range of low doses, without changes in the same direction for several higher doses. This can be seen for exposure to both permethrin and the organochlorine heptachlor (Karen et al. 2001; Bloomquist et al. 2002).

The present immunohistochemical data, combined with previous kinetic data, suggest that pyrethroid insecticides are capable of altering the normal functional status of the nigrostriatal pathway. The tissue damage suggested by increased GFAP within the caudate-putamen has obvious implications for the putative connection between pesticide exposure and PD. However, alterations of DAT protein within this region may also represent an important substrate modulating the onset and severity

of Parkinson's disease, because this transporter is the means by which Parkinson's disease-like inducing chemicals (e.g. MPP<sup>+</sup>) can enter dopaminergic neurons (Mayer, Kindt, and Heikkila 1986; Gainetdinov et al. 1997; Bezard et al. 1999). Such changes in the integrity of the DAT may be important for future investigations of the role of synergistic interactions between environmental chemicals in the development of PD.

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